The Role of the Second Subunit of the Origin Recognition Complex (ORC2) in the Development of *Arabidopsis thaliana*

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ZUSAMMENFASSUNG

Der Origin Recognition Complex (ORC) spielt eine wichtige Rolle bei der Initiierung der DNA Replikation durch Bindung an die Replikationsstartstelle und Rekrutierung der Replikationsmaschinerie. Im Modellorganismus *Arabidopsis thaliana* führt eine Mutation in der zweiten Untereinheit des ORC (ORC2) zu einem für einen Replikationsmutanten unerwarteten Phänotypen: Embryonen entwickeln sich abnormal, und das Endosperm, Nährgewebe für den Embryo, besteht aus gigantischen Zellkernen mit drastisch erhöhtem DNA-Gehalt. In der vorliegenden Arbeit wird ein auf der detaillierten Charakterisierung des ORC2-Mutanten basierendes Modell vorgeschlagen, um diese speziellen Phänotypen auch bei anderen Replikationsmutanten zu erklären.

ABSTRACT

The Origin Recognition Complex (ORC) is composed of six subunits that are conserved throughout eukaryotes. It was originally discovered in yeasts as a protein complex that acts in replication initiation by binding replication origins and recruiting the replication machinery to the DNA. *Arabidopsis* plants, mutant for the second subunit of the complex (the ORC2 protein) are not viable and abort early in embryogenesis. Aborting embryos are disorganized and the endosperm, an extraembryonic tissue, contains dramatically enlarged nuclei, a phenotype that is not intuitively expected for a mutant in a replication protein. This work represents an attempt to understand this discrepancy. Extensive analysis of *ORC2* expression and of the phenotype of three *orc2* mutant alleles was performed. The results of this analysis allowed me to propose a model explaining why this mutation leads to the observed phenotype. Also, a system allowing RNAi-mediated inducible knockdowns was developed, as well as a system to perform a suppressor screen for modifiers of the *orc2* mutant phenotype.

To my aunt, Zoya Goltiaeva

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1. INTRODUCTION

A line (ET3753) that exhibited zygotic lethality was identified in a screen for semisterility in a collection of enhancer-trap *Ds* transposants (Moore et al., 1997). The transposable element disrupted the *AtORC2* gene (encoding the second subunit of the Origin Recognition Complex, ORC), and this insertion was shown to cause the phenotype. In this line, mutant embryos arrest at the preglobular stage, and are disorganized. The endosperm in mutant seeds proceeds only through 2 to 3 rounds of division and the nuclei are dramatically enlarged compared to wild-type and have a giant nucleolus (Collinge et al., 2004). The appearance of large nuclei is unexpected and has not been observed in ORC mutants in other organisms. This work represents an attempt to understand why the *orc2* mutation leads to such phenotypes in *Arabidopsis*, and what is the role of the ORC2 protein in *Arabidopsis* development.

Since its discovery 15 years ago (Bell SP, 1992), ORC attracted significant attention in the scientific community, for being not only a central element in eukaryotic replication initiation, but also an essential component of a variety of chromatin-related cellular processes. The Introduction chapter of this thesis summarizes what is known about ORC functions in eukaryotes, and reviews the main aspects of seed development in *Arabidopsis thaliana*. This knowledge was essential for the establishment of the model for the observed *Arabidopsis orc2* mutant phenotype presented in chapters 4.1 and 4.2 of this manuscript.

1.1. The variety of plant life cycles

From the evolutionary point of view, the ultimate goal of every living creature is to preserve and to successfully pass its genome on to the next generation. Plants and animals deal with this task in very different ways. Animals differentiate the germline (cell lineage that produces gametes) very early in their development. The reason for this is clear: every genomic replication event generates new mutations, so the fewer cell divisions occurring before a gamete is formed, the fewer mutations will be transferred to the progeny. Plants employ a different strategy: they do not produce a germline, but alternate between a haploid generation, the gametophyte, and the diploid, the sporophyte (Figure 1-1) (reviewed in (Walbot and Evans, 2003).

The existence of a haploid generation ensures that deleterious mutations are not transmitted to the following generation. If an essential gene that is present in a single copy is damaged, the gametophyte will not survive. Although the advantages of the haploid generation are clear, there is a steady trend for the reduction of the importance of the haploid generation throughout plant evolution. In green algae and in mosses the haploid stage comprises the majority of the life cycle, but already in ferns the sporophytic stage becomes dominant. In seed plants, gametophytes are unable to photosynthesize and are completely dependent on the sporophyte for their nutrition. The sexual process results in the formation of a specialized structure, the seed, which contains the diploid embryo and a nourishing tissue, the endosperm, which provides nutrients to the growing embryo.

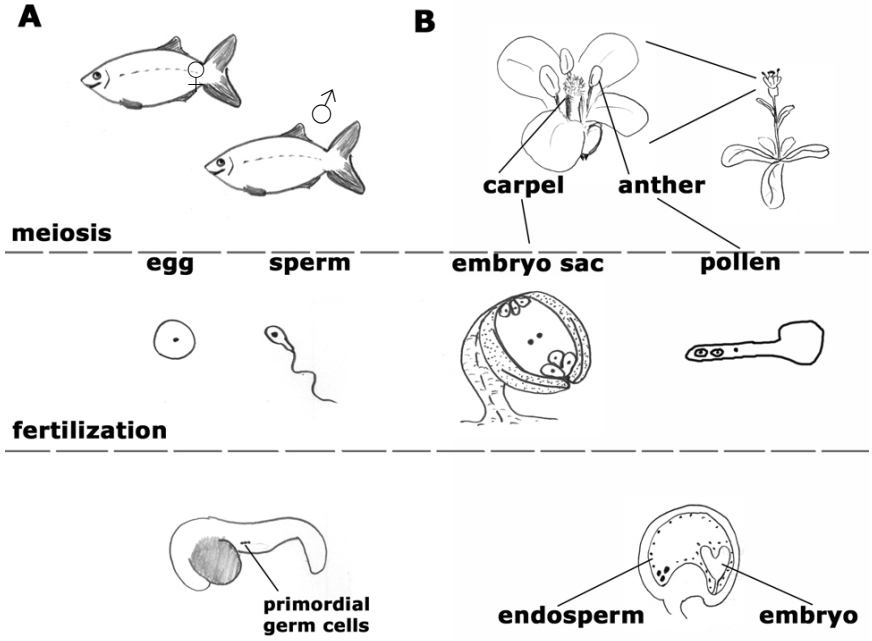


Figure 1-1. Animal and plant life cycles. A. In animal life cycles gametes are direct meiotic products. Meiosis occurs only in the germline – a cell lineage that is differentiated very early in embryogenesis (primordial germ cells). **B**. In plants, male and female sexual organs are produced late in development. Meiotic products undergo a number of mitotic divisions and form haploid multicellular organisms – the female and male gametophytes. (Figure modified from (Walbot and Evans, 2003)).

1.2 Seed development in angiosperms

Seed development in flowering plants has a number of unique features. Each gametophyte produces two gametes: the pollen (male gametophyte) two sperm, and the female gametophyte an egg cell and the central cell. Seed formation is then the result of a double fertilization event (Nawaschin, 1898; Guignard, 1899). One of the

sperm cells fertilizes the egg cell, producing the embryo, while the other fuses with the central cell. The central cell is typically diploid and the karyogamy between its nucleus and the haploid sperm nucleus produces triploid endosperm. Together with integuments, which are sporophytically derived, the embryo and the endosperm form the seed of flowering plants. The endosperm acquires nutrients from the mother plant and provides them to the developing embryo, making it an essential link within the life cycle of angiosperms. Before proceeding to review seed development in *Arabidopsis*, gametophyte development in angiosperms will be briefly reviewed.

1.3 The structure and development of the Polygonum*-type female gametophyte*

The development of the female gametophyte (megagametogenesis) in most angiosperm species starts with the formation of a primordium, consisting of three layers (LI, LII and LIII) (reviewed in (Koltunow and Grossniklaus, 2003)). The sporogenic tissue develops within the nucellus, which originates from the LII layer. Within the nucellus, usually a single Megaspore Mother Cell (MMC) differentiates. The MMC is larger than the surrounding cells, occupying much of the space in the developing ovule (Bajon et al., 1999). The MMC undergoes meiotic reduction, resulting in the formation of four megaspores. The number of megaspores that later form female gametophytes is variable among angiosperms (Willemse and van Went, 1984), but the *Polygonum*-type embryo sac, which is the most frequent in angiosperms and found in *Arabidopsis* and cereals, is formed from a single haploid spore, while the other three degrade. This haploid cell, the functional megaspore, undergoes three mitotic divisions without cytokinesis, to form a syncytium. After the first division the two nuclei migrate to the opposite poles of the embryo sac, so that the subsequent two rounds of mitosis produce 2 groups of 4 nuclei. Cell membranes are formed around three nuclei at each pole, while one nucleus from each group (termed the polar nuclei) migrates to the middle of the embryo sac (Figure 1-2). Thus, the *Polygonum*-type embryo sac consists of seven cells bearing eight nuclei (Willemse and van Went, 1984).

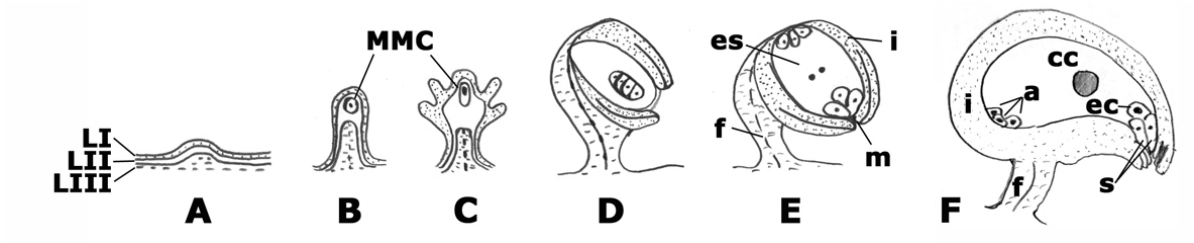


Figure 1-2. Development of the *Polygonum*-type female gametophyte. A. The ovule primordium is composed of three layers. **B.** Cells within the LII layer produce sporogenic tissue, where a Megaspore Mother Cell (MMC) differentiates. **C.** Integuments develop as an outgrowth of the LI layer. **D.** MMC undergoes meiosis to produce 4 megaspores. **E.** One of the megaspores undergoes three mitotic divisions to produce the embryo sac (es). Integuments (i) surround the embryo sac almost completely, leaving a narrow aperture, the micropyle, at the tip of the ovule. Funiculus (f) connects the ovule to the maternal tissue. **F.** In *Arabidopsis*, the mature embryo sac consists of seven cells: 2 synergids (s) adjacent to the micropyle, the egg cell (ec), the central cell (cc), and 3 antipodals (a) at the chalazal end of the ovule (figure modified from (Koltunow and Grossniklaus, 2003)).

Parallel to embryo sac development, the LI layer cells also start to divide, forming the three layers of integuments that surround the embryo sac. In most species, integuments cover the ovule almost completely, but are not fused at the tip, leaving a channel, the micropyle, for a pollen tube to enter the ovule. At the micropylar end of the embryo sac lie three cells: the egg cell and two synergids that participate in pollen tube attraction and reception (reviewed in (Higashiyama et al., 2001). At the opposite pole of the ovule, the chalazal end, are three antipodal cells. The two polar nuclei fuse together to form the diploid central cell.

1.4 Arabidopsis *male gametophyte, pollen*

Pollen development (microsporogenesis) in *Arabidopsis* occurs within anthers, where haploid microspores are formed as the result of meiosis. The first division of the microspore nucleus produces two cells: a small generative cell with highly compacted chromatin and a large vegetative cell. The generative cell undergoes another mitotic division to produce two sperm cells that are surrounded by the cytoplasm of the vegetative cell. In *Arabidopsis*, the generative cell divides prior to anther dehiscence and anthesis. Importantly, almost immediately after mitosis, S-

phase is initiated in sperm cells, so that at anthesis sperm nuclei have an approx 1.5n DNA content (Friedman, 1999).

Thus, a mature pollen grain of *Arabidopsis* deposited on the stigma comprises three cells: two sperm cells and one large vegetative cell that surrounds the sperm cells and performs the growth of the pollen tube within the flower to reach the ovule (reviewed in (Boavida et al., 2005)). As the pollen tube enters the ovule, sperm cells are released to fertilize the egg and central cells.

1.5 Fertilization

In most eukaryotes karyogamy to produce the zygote occurs between two haploid nuclei that are in the G1 phase of the cell cycle. However, within seed plants an alternative strategy also exists. Nuclear fusion can happen within the G1 phase, as in barley (Mogensen and Holm, 1995) and maize (Mogensen et al., 1995), or in G2 as has been reported for Gnetum (Carmichael and Friedman, 1995), Arabidopsis (Friedman, 1999) and tobacco (Tian et al., 2005). Also, a peculiar karyogamy type was reported to exist in Ephedra, where gametes after the fusion do not proceed to karyogamy, but first pass through S-phase duplicating their DNA content (Friedman, 1991).

1.6 Endosperm development in Arabidopsis thaliana

As described above, the embryo sac in *Arabidopsis* is of a *Polygonum*-type and fertilization triggers seed development. The endosperm starts to proliferate shortly after fertilization and its proliferation also triggers the proliferation of the seed coat (Ingouff et al., 2006). Early endosperm divisions occur without cytokinesis, leading to development of a syncytium. Syncytia are commonly observed in plant endosperms, but are not unique. In *Drosophila*, the first 13 divisions of the zygote also occur without cytokinesis and proceed very rapidly. Importantly, mitoses in syncytia occur synchronously, and *Arabidopsis* endosperm is not an exception. A careful cytogenetic study showed that the first three divisions of the central cell in *Arabidopsis* occur synchronously, and at this time point three mitotic domains within

the endosperm are established, in which nuclei either go through coordinated divisions or increase their size (Boisnard-Lorig et al., 2001). These domains are the micropylar endosperm (MCE) that surrounds the embryo, the peripheral endosperm (PEN) that comprises the majority of the endosperm, and the chalazal endosperm (CZE).

The synchronicity of divisions in the PEN and MCE continues up to the fifth division. Then starting from the sixth cycle, a wave of mitotic divisions crossing the PEN from the micropylar to the chalazal end is observed (Boisnard-Lorig et al., 2001). Early divisions in both MCE and PEN occur rapidly, every 20-30 minutes. The CZE nuclei do not divide, and their number in a single seed varies between 1 and 4. However, their ploidy level reaches 24n, in contrast to the maximal 6n observed in PEN, and this higher ploidy level is the result not only of endoreduplication (consecutive S-phases without division), but also of nuclear fusion (Baroux et al., 2004). Later in seed development cell walls are formed around endosperm nuclei, and their mitotic activity ceases. As the *Arabidopsis* embryo matures, it consumes the endosperm, and terminally developed seed contains the embryo and only a single layer of endosperm cells.

It must be emphasized, that endosperm development substantially differs from that of other plant tissues, especially with the respect to the cell cycle. First, endosperm nuclei undergo endoreduplication, to maximum 24n in *Arabidopsis*, but their ploidy can reach values as high as 690C in the endosperm of some maize cultivars (Lopes and Larkins, 1993). The endocycle is often considered as a simplified version of the mitotic cycle. The mitotic cycle consists of G1, S, G2 and M phases, while the endocycle is composed of two phases: S and a Gap phase. In all eucaryotes the progression of the cell cycle is controlled by sequential activities of Cyclin-Dependent Kinases (CDKs) that regulate the timing and substrate specificity by interacting with different cyclin proteins (Francis, 2007). The removal of the M-phase requires deactivation of some components of the cell cycle machinery. Deactivation of Cyclin E was shown to be important for the switch to endocycles in both animals (Vidwans et al., 2002) and plants (Roudier et al., 2000). Also, at least in animals, endocycling cells have altered checkpoint functions, which makes them more tolerant to DNA damage (MacAuley et al., 1998). Although it has not been directly shown in plants, it is likely that checkpoint functions are also modified in endocycling plant cells. Another distinctive feature of *Arabidopsis* endosperm development, is that most divisions

occur without the formation of cell membranes. And finally, nuclear fusions occur in the chalazal endosperm (preceded by nuclear migration (Guitton et al., 2004)), a process that has been described in a very few cases among living organisms (of course, with the exception of karyogamy at fertilization). Overall, these facts strongly argue that the cell cycle machinery is modified in the endosperm, and some components of the cell cycle are not active.

1.7 Embryo development in Arabidopsis thaliana

The *Arabidopsis* embryo develops from the fertilized egg cell. Shortly after fertilization the zygote starts to elongate and becomes polarized. Its divisions are delayed in comparison to the endosperm: the zygote performs its first division at the time when endosperm has already gone through two to three divisions. The first division of the zygote occurs transversely and is asymmetric: it produces a small apical cell and an elongated basal cell. The apical cell will give rise to the entire embryo, except for a few cells of the root primordium. The basal cell gives rise to the suspensor, an extraembryonic tissue that transfers nutrients from the mother plant to the embryo; and the most distal cell, the hypophysis, which produces the quiescent centre and columella of the root tip. Unlike the strictly determined divisions of the apical cell lineage, the basal cell divisions are less strictly defined. Before the first division of the apical cell, the basal cell may divide once or twice, or not divide at all.

The first division of the apical cell is longitudal and produces two cells that later divide longitudally to produce the quadrant embryo. Each of these cells undergoes a transverse division resulting in an octant stage embryo. At this point the apical and the basal domains of the embryo are formed (Jurgens, 1992). It is worth noting that, although these early divisions are not strictly synchronous, they are tightly coordinated, as their importance for the establishment of basic embryo axes is great, and wrong timing of these divisions can theoretically affect embryo morphology. The next round of divisions produces two layers of cells – the outer (the protoderm), which will give rise to the epidermis, and the inner, that will produce procambium and ground tissue. At this stage (dermatogen), the radial pattern is formed. Later, as the embryo proceeds to the globular stage, the cells of the inner layer divide longitudally, and the protoderm cells divide anticlinally. At this stage the synchronicity of divisions in the embryo proper is lost.

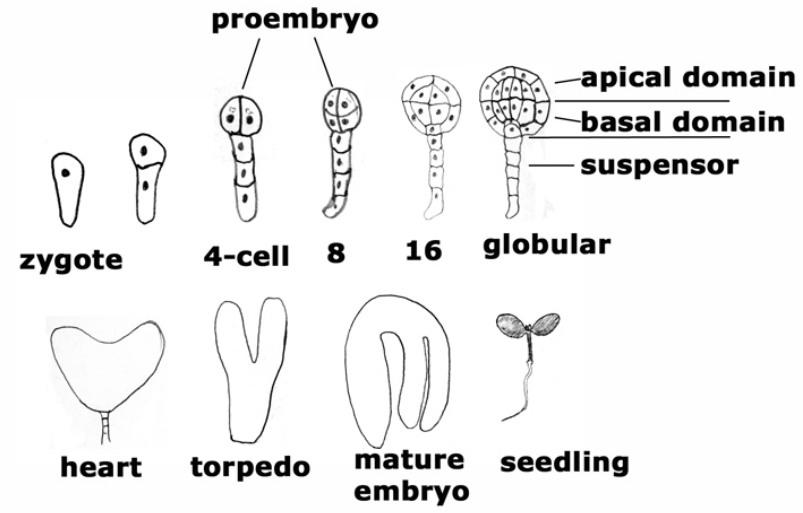


Figure 1-3. Embryo development in *Arabidopsis*. Stages of *Arabidopsis* embryo development. Up to globular stage cells divide in an invariable pattern. At the 8-cell stage the apical and basal domains of proembryo are established.

At the globular stage the development of the suspensor is essentially accomplished. It consists of 7-9 highly vacuolated cells at the bottom. The cell at the top, immediately adjacent to the embryo, is called the hypophyseal cell, and it acquires a distinct shape. This cell will give rise to the root meristem and the root cap. At the late globular stage cell divisions at the positions of the future cotyledons accelerate, forming a triangular embryo. At this stage all the basic embryo patterns are formed (Jürgens et al., 1995). Rapid divisions in cotyledon primordia result in the formation of the heart-stage embryo. Further divisions in the cotyledon primordia, as well as cell elongation in the radicle and hypocotyls, produce the torpedo embryo. Further growth of the embryo makes it bend (walking stick stage), and at the maturity the embryo occupies the whole seed volume.

The critical stages in embryo development are those that establish the body axes. These are the first zygotic division, the transitions from quartet to octet, octet to dermatogen, and globular to triangle. Prior to the triangle-heart stage, cell divisions follow a strict developmental pattern, which, when disturbed, in most cases results in seed abortion. Embryos bearing mutations affecting later stages of embryo development can, in most cases, be successfully rescued by removing them from the seed, if they have reached the triangle stage (Franzmann et al., 1989).

My project was centered on the *Arabidopsis orc2* mutant. Embryos, homozygous for the mutation, abort at preglobular stage and appear disorganized (Collinge et al., 2004). The analysis presented in Chapter 3.1 shows that embryo patterning abnormalities start to appear as early as the second division of the zygote. A description of the known functions of the ORC2 protein in other organisms, and

Arabidopsis mutants that exhibit similar phenotypes, is presented in the following chapter.

1.8 Eukaryotic Origin Recognition Complex (ORC)

Precise and complete replication of the nuclear DNA is an integral part of the cell cycle. In dividing eukaryotic cells, the nuclear DNA has to be replicated once and only once per cell cycle. The Origin Recognition Complex (ORC) plays a central role in the initiation of nuclear DNA replication. The ORC is composed of six subunits (ORC1 to ORC6) that are conserved throughout eukaryotes (reviewed in (Bell, 2002)). ORC binds origins of DNA replication prior to the S phase of the cell cycle and recruits the Cdc6p and Cdt1p proteins. These proteins, together with the Mcm2-7 complex, the putative replicative DNA helicase, constitute the prereplicative complex (PreRC) (reviewed in (DePamphilis, 2005)). Once the preRC has been assembled, the replication machinery can be recruited to the DNA.

Once replication has started from the origin, the Pre-RC has to be inactivated to avoid replicating the DNA from the same origin twice within a single S-phase. This inactivation of the Pre-RC is achieved in different ways between species. In *Saccharomyces cerevisiae,* ORC2 and ORC6 are phosphorylated after replication has been initiated (Nguyen et al., 2001). Higher eukaryotes employ further controls, including physical removal of ORC subunits from the chromatin after the replication has started. In *Drosophila*, The ORC2-6 complex stays associated with the chromatin throughout the cell cycle, while ORC1 dissociates from the chromatin in the S phase (Asano and Wharton, 1999), and is degraded in an Anaphase-Promoting Complex (APC) dependent manner (Araki et al., 2003) Similarly in mammalian cells, the core ORC subunits, ORC2-5, are bound to chromatin throughout the cell cycle, at least in a number of cell lines (Dhar and Dutta, 2000; Tatsumi et al., 2003), while ORC1 only accumulates and binds to chromatin during G1 phase (Ohta et al., 2003; Tatsumi et al., 2003). HsOrc1 is degraded by ubiquitin-dependent proteolysis in S-phase (Mendez et al., 2002), but Chinese hamster ovary cells mono-ubiquinate Orc1 resulting in its sequestration in the cytoplasm (reviewed in (DePamphilis et al., 2006). In activated egg extracts of the amphibian *Xenopus laevis*, the whole ORC dissociates from the somatic cell chromatin (Sun et al., 2002). Interestingly, ORC does not dissociate from sperm chromatin in *Xenopus* (reviewed in (DePamphilis, 2005)). It is remarkable that even within a group of closely related organisms, the

vertebrates, such a variety of strategies to prevent re-replication exists. This fact further highlights the importance of its prevention, and suggests that pathways leading to ORC relocation during the cell cycle might have arisen independently several times during evolution.

In addition to its role in nuclear DNA replication, ORC has been shown to play a role in silencing and heterochromatin structure. In yeast cells the ORC is important for silencing of mating-type loci (Bell et al., 1993; Micklem G, 1993): it recruits the Sir1p protein (Triolo and Sternglanz, 1996), the first member of the complex changing the chromatin structure around these loci. In *Drosophila*, mutations in *DmOrc2* lead to mislocalization of the Heterochromatin Protein 1 (HP1) on the chromatin (Shareef et al., 2001) and to suppression of Position Effect Variegation (Pak et al., 1997), a heterochromatin-mediated silencing phenomenon. Components of the ORC were shown to interact with HP1 in mammalian cells (Auth et al., 2006). In addition to the role in maintenance of constitutive heterochromatin, ORC was also shown to be involved in the processes of mitotic chromosome condensation. In *DmOrc2, 3* and *5* mutants metaphase chromosomes appear thicker and shorter than in the wild type (Pflumm MF, 2001).

However, it is possible that abnormal condensation of mitotic chromosomes has a direct link to the replicative function of the ORC2 protein. An elegant model directly connecting replication and chromosome condensation has been proposed (reviewed in (Pflumm, 2002)). This model assumes that two replication forks from one origin are connected to each other, so that replicated strands of DNA emerge from the complex of two replicative forks as two loops. Secondly, this model utilizes the notion that chromatid cohesion and compaction is established co-replicatively. From these assumptions it follows that the frequency and the length of replicons would influence chromosome compaction, i.e. the more rare replicons are, the bigger will be the loops emerging from the DNA replication site, and hence, the thicker and longer the condensed chromosome will appear. The phenotype observed in ORC mutants in *Drosophila* supports this theory (Pflumm MF, 2001).

Notably, in metazoan cells lacking ORC two phenotypes are usually observed. The first class consists of cells that are blocked at the G1/S transition, and the second of cells that are not able to complete mitosis and are blocked in M-phase with abnormally condensed chromosomes (Dillin and Rine, 1998; Loupart ML, 2000;

Pflumm MF, 2001). This raised the possibility of an additional role for ORC in mitosis or in chromosome cohesion and condensation, but it was argued that M-phase arrest could arise solely from aberrant DNA replication. It is only comparatively recently that evidence for ORC subunit functions in mitotic processes has been reported. In yeast, ORC has now been shown to be involved in sister chromatid cohesion (Suter et al., 2004; Shimada and Gasser, 2007) In animals, it is important at the interphase to mitosis transition, to disassemble Replication Protein A (RPA) foci (Cuvier et al., 2006). ORC subunits bind to centrosomes in mammalian cells showing they may have a role in chromosome segregation (Prasanth et al., 2004; Stuermer et al., 2007). Lastly, two ORC subunits have been linked to cytokinesis. The detection of a pool of ORC6 outside the nucleus led to the discovery that it functions outside the ORC at kinetochores and in cytokinesis (Prasanth et al., 2002; Chesnokov et al., 2003). ORC2 is at the mid-body of mouse cells at cytokinesis, and its depletion leads to multinucleate cells (Stuermer et al., 2007). That ORC functions in M-phase and cytokinesis, is now clear, but precisely what it does and how it is regulated is still largely unknown.

Research focused on ORC currently moves in three general directions: identification of novel members of the PreRC, identification of novel ORC functions, and attempts to identify what marks a stretch of DNA as an origin of replication in higher eukaryotes.

Plant genes encoding ORC homologues have been characterized (Witmer et al., 2003; Masuda et al., 2004; Diaz-Trivino et al., 2005; Mori et al., 2005), but very little is known about the ORC function in plants. A previous study of an ORC2 mutant in *Arabidopsis* revealed that the *AtORC2* gene is essential for development, and plants homozygous for the *orc2* mutation die very early in embryogenesis (Collinge et al., 2004). Also, a genetic interaction was shown between the AtORC2 and a *Polycomb*-group protein, MEDEA. Proteins of this family are known to be involved in processes of silencing and establishment of chromosome structure (reviewed in (Guitton and Berger, 2005)). The phenotype caused by the *orc2* mutation was, in part, unexpected. Embryos had abnormal patterning, and the endosperm contained dramatically enlarged nuclei, which was surprising considering that the replication function was likely affected by the *orc2* mutation.

1.9 Arabidopsis *mutants exhibiting similar phenotypes*

Interestingly, a similar phenotype has also been observed in other *Arabidopsis* mutants. Seeds of embryo-lethal *pilz* (Steinborn et al., 2002) and *titan* (*ttn*) (Tzafrir et al., 2002) class mutants also contain giant endosperm nuclei. Genes, disrupted by *pilz* mutations, as well as *ttn1* and *ttn5* encode tubulin folding cofactors, and *titans* 3, 7 and 8 encode homologues of condensin and cohesin, proteins important for chromosome compaction. Enlarged endosperm nuclei have also been observed in the *prolifera (prl)* mutant in a homolog of the MCM7 gene (Holding and Springer, 2002), encoding a member of the putative replicative DNA helicase complex, and in mutants for subunits of a replicative DNA polymerase ε (Jenik et al., 2005; Ronceret et al., 2005). Collectively, these data suggest that the enlarged endosperm nuclei phenotype can be caused either by disruption of cytoskeletal machinery, or if the nuclear DNA replication or condensation is affected. In the case of cytoskeletal machinery and chromosome cohesion/condensation mutants, the link between the gene function and the observed phenotype can be intuitively established. These mutations affect proteins that are directly involved in mitotic processes, rendering the increase in nuclear size in these mutants not surprising. However, the fact that the giant endosperm nuclei are observed also in mutants for cell cycle components (*TILTED1*, *PROLIFERA*, *TTN6*, *ORC2*, 26S proteasome subunits (Brukhin et al., 2005) and many others), is surprising, and a model explaining this fact has not been described yet. In the Results and the Discussion chapters of this manuscript, I present a more extended review of the aforementioned mutants, and put forward a hypothesis attempting to explain these phenotypes.

1.10 Aims of the project

The aim of this thesis is to shed light on the role of ORC2 in *Arabidopsis* development. To get insights into its functions in this plant model system, at the onset of this project, the following aims were set:

- Attempt to identify partners of ORC2 protein in *Arabidopsis* by performing a suppressor screen.
- Perform a thorough characterization of the *orc2* mutant that can bring clues to understanding how the mutation leads to the observed phenotype.
    - Characterize the embryo phenotype
    - Characterize the chromatin structure in the giant endosperm nuclei of the *orc2* mutant
    - Propose a model explaining the observed phenotype
- Attempt to knock down ORC2 expression in adult tissues to investigate its possible function in post-embryonic development.
- Identify the intracellular localization of the ORC2 protein, preferably throughout the cell cycle.

2. MATERIALS AND METHODS.

2.1 Plant material and growth conditions.

Arabidopsis thaliana (L.) Heynh., accession Landsberg *erecta* (*Ler*) was used as a wild-type unless indicated otherwise. The *orc2-1* mutant (ET3723) was isolated in a screen for semisterility in a collection of enhancer trap *Ds* transposants (Moore et al., 1997). The *orc2-2* line (SALK_027788) was obtained from the SALK collection of T-DNA insertional mutants (Alonso et al., 2003). The *orc2-3* mutant (GT7766) was identified in a collection of gene trap *Ds* transposants (Martienssen, 1998) and kindly provided by Robert Martienssen. The following non-AtORC2 mutants were obtained from the stock centre: *ttn2 (TITAN2)* (Liu and Meinke, 1998), *ttn4, ttn8* (Tzafrir et al., 2002), *fis3 (fie) (FERTILIZATION INDEPENDENT SEED 3 (FERTILIZATION INDEPENDENT ENDOSPERM))* (Ohad et al., 1996), *eza1* (Mayama et al., 2003), *tfl2 (TERMINAL FLOWER 2)* (Larsson et al., 1998). The *msi1-2* line (mutant for the *Arabidopsis* homologue of yeast gene *MULTICOPY SUPPRESSOR OF IRA-1*) (Guitton et al., 2004) was kindly provided by Fréderic Berger. The *fis2* line *(FERTILIZATION INDEPENDENT SEED 2)* (Chaudhury et al., 1997) was kindly provided by Abed Chaudhury. The Arabidopsis line expressing the *CycB1*;1::GUS:DB construct (Colon-Carmona et al., 1999) was acquired from Vladimir Brukhin.

Seeds were surface-sterilized with 2% sodium hypochlorite and were allowed to germinate on MS medium (Duchefa, Haarlem, The Netherlands) containing 10g/L sucrose and 8 g/L of agar. To select plants, containing *Ds* or T-DNA insertions, the corresponding antibiotic was added to the medium. The selection against plants carrying the CodA gene was performed on MS media containing 1mg/ml 5-fluorocytosine (5-FC) (Fluka AG, Buchs, Switzerland). For the inducible RNAi experiments, seedlings were grown for 10 days on MS media, and then induced by transplanting to MS medium containing 10µg/ml estradiol and 0.01% ethanol (or 0.01% ethanol alone, as control).

Two-week old seedlings were transplanted to ED73 soil (Tränkle Einheiteserde, Kappelrodeck-Waldulm, Germany) that was covered with 3mm of sand and initially irrigated with water containing 0.6 g/l nematodes (Traunem *Steinernema felitiae*, Andermatt Biocontrol AG, Switzerland), and transferred to a growth chamber with

70% humidity and a day/night cycle of 16 h light at 21°C and 8 h dark at 18°C. Selection of BASTA-resistant plants was performed on soil by spraying the plants with 0.05% v/v BASTA (Plüss&Stauffer AG/SA, Oftringen, Switzerland) twice with a three-day interval between treatments.

2.2 Standard molecular biology protocols and reagents

All standard molecular biology procedures were performed as described in (Sambrook and Russel, 2001) or as described in the manufacturer's protocol if a kit was used. Chemicals were purchased from Sigma (Sigma-Aldrich Inc., St. Luis MO, USA) if not specified otherwise. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and corresponding enzyme buffers were obtained from New England Biolabs (Ipswich, MA, USA). The Taq polymerase was acquired from Sigma (Sigma-Aldrich Inc., St. Luis MO, USA), high fidelity *Taq* polymerase, DNAseI was obtained from F. Hoffmann-La Roche AG (Basel, Switzerland). Trizol reagent, Superscript II, 0.1M DTT, oligo-dT primer and RNAse OUT reagent were purchased from Invitrogen Corporation (Carlsbad CA, USA). Plasmid DNA purification was performed using GFX PCR DNA and Gel Band Purification Kit (Amersham Health, Braunschweig, Germany). PCR products were purified using the Qiagen PCR Purification Kit and purification of DNA bands from agarose gels was carried out using the Qiagen MinElute Gel Extraction Kit (Qiagen AG, Hombrechtikon, Switzerland). Small-scale plasmid isolations (mini-preps, 1.5 ml culture) were performed by alkaline lysis and the DNA was stored in 40µl TE pH 8.0. Large-scale plasmid isolations (midi-preps) were performed with the JETSTAR Plasmid Purification Kit (Genomed, Löhne, Germany). The Gateway^{TM} cloning system was supplied by Invitrogen Corporation (Carlsbad CA, USA), recombination reactions were performed according to the manufacturers instructions except that all reaction volumes were halved.

2.3 RNA extraction and RT-PCR

RNA was extracted using Trizol reagent according to the manufacturer's instructions. 5µg of total RNA was treated with DNaseI for 30 minutes at 37° in the presence of RNAse OUT reagent, and the reaction was stopped by phenol-

chloroform extraction. The DNA-free RNA was then ethanol-precipitated from the aqueous phase and dissolved in DEPC-treated water. For Reverse Transcription reactions, 1µg of DNAseI-treated RNA was mixed with 1µl of 0.5µg/ml oligo-dT primer and 1µl RNAse OUT reagent in a final volume of 15µl. The solution was incubated at 70°C for 10 minutes to denature RNA and primers, and then cooled on ice. The RNA/primer mix was supplemented with 8µl 5x first-strand buffer, 0.8µl 0.1M DTT, 1.6µl 25mM dNTPs and DEPC-treated water in a final volume of 39µl. 20µl of this mix was taken to a separate tube as a control for genomic DNA contamination, and to the remaining 19µl 1µl of Supercript II reverse transcriptase was added. The samples were incubated at 42°C for 60 minutes, and stopped by incubating at 72°C for 20 minutes.

For PCR amplification 1µl of the reverse transcribed mix was taken per reaction. Reactions were performed in following conditions: 50mM KCl, 1.5mM $MgCl_2$, 10mM TRIS-HCl, pH 9.0, 0.2mM dNTPs, 0.5µM forward and reverse primers (for primers used see Results and Supplementary Material), 2U Taq polymerase in 20µl reaction volume. The annealing temperature (T_a) was set 10°C below the oligonucleotide melting temperature (T_m) indicated by the manufacturer (SIGMA Genosys, Riedstrasse 2, 89555 Steinheim). For the extension time (t_e), a synthesis rate of one kilobase (kb) per minute (1 $kbmin^{-1}$) was assumed and the relevant elongation phase duration was calculated. PCR reactions were performed using a PTC-200 thermocycler (MJ Research, Waltham MA, USA), starting with an initial denaturation step at 95°C for 2 min followed by 20-40 cycles of 15 sec denaturation at 94°C, annealing phase of 10 sec and elongation phase at 72°C. An additional two minutes of elongation at 72°C followed the last cycle. For semi-quantitative RT-PCR, four replicas were set for each reaction, and reactions were interrupted after 21, 25, 28 and 35 cycles of amplification to ensure that the amplification of the product did not reach plateau.

For the analysis of ORC2 transcript in *orc2* mutant alleles, RNA was purified from 1-DAP siliques, and for the RNA analysis in the Inducible-RNAi lines, RNA was purified from whole seedlings.

2.4 Bacterial strains, media and transformation

Escherichia coli strains DH5^{TM} (F^- *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) $_{sup}$E44 λ^- *thi*-1 *gyr*A96 *rel*A1) and DB3.1^{TM} (F- *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20 (r_B^-, m_B^-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 Δ*leu mtl*1) were used for cloning and propagating plasmid constructs. Transformation of chemo-competent *E. coli* was performed following the calcium chloride (heat-shock) method. Frozen 100 µl aliquots of competent cells were stored at -80°C. *E. coli* strains were grown at 37°C in *Luria Bertani* (LB) liquid medium or on LB-plates (LB medium and 1.8% w/v Bacto Agar, Chemie Brunschwig AG, Basel, Switzerland) with antibiotics if required [100 µg/ml spectinomycin (spec), Duchefa Biochemie RV, Haarlem, Netherlands; 50 µg/ml ampicillin (amp) and 50 µg/ml kanamycin (kan), both AppliChem, Darmstadt, Germany; 10 µg/ml gentamicin sulphate (gent), Fluka AG, Buchs, Switzerland; 25 µg/ml chloramphenicol].

For plant transformations *Agrobacterium tumefaciens* strain GV3101 containing the helper plasmid pMP90 was used (Koncz and Schell, 1986). This strain is gentamicin (40µg/ml gentamicin sulfate, Fluka AG, Buchs, Switzerland) and rifampicin resistant (50 µg/ml rifampicin). Additional antibiotics, where needed, were added as described above. *A. tumefaciens* cells competent for plasmid transformation grew as 25 ml LB or YEP cultures (YEP: 10 g/l yeast extract, 10 g/l tryptone peptone, 5 g/l NaCl, pH 7.0) overnight at 28°C and were harvested by centrifugation at 4000 rcf for 10 min. The resulting pellet was re-suspended in 1 ml ice-cold 20 mM $CaCl_2$ and aliquots of 100 ml were shock frozen in liquid nitrogen and stored at –80°C. *A. tumefaciens* transformation was carried out by adding 1-2 mg plasmid DNA to a frozen aliquot and incubating for 5 min at 37°C. Then 1 ml LB was added and the samples were incubated for 2 h at 28°C before transfer onto LB plates containing the required antibiotics. Colonies were picked after 4 d incubation at 28°C. To confirm the identity of plasmids in *A. tumefaciens*, plasmid mini-prep was carried out followed by re-transformation of *E.coli* DH5α^{TM} cells for control analyses by restriction digest and/or sequencing. Large cultures (250- 500 ml) of the GV3101 strain for plant transformation routinely contained gentamicin, and were inoculated with 5 ml of an overnight preculture containing gentamicin and rifampicin.

Plant transformations were performed using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Transformations were performed on plants 7-10 days after the production of the primary bolt. A 250-500 ml liquid culture of *A. tumefaciens* strain GV3101 carrying the required plasmids was grown for 20 h at 28°C with shaking at 180-200 rpm. Cells were pelleted by centrifugation (Beckmann J2-21 Centrifuge and JA-10 rotor, 5000 rpm for 10 min; Beckmann Coulter, Krefeld, Germany) and resuspended to OD600=0.8 in transformation medium (5% w/v sucrose, AppliChem, Darmstadt, Germany; and 0.02% v/v Silwet L-77, Lehle Seeds, Round Rock TX, USA). Freshly dipped plants were covered for 24 h.

2.5 Whole-mount preparations, histology and cytology.

For morphological analysis of developing seeds, siliques were fixed and cleared in chloral hydrate (Yadegari et al., 1994). Siliques were dissected, mounted in clearing solution and analyzed with a Leica DMR microscope using bright-field Nomarski optics. Specimens of ORC2:GFP expressing plants were fixed in 4% formaldehyde, washed twice with distilled water, mounted in a medium containing 1 M glycine and 1 ug/ml DAPI and inspected with a TCS SP2 confocal laser scanning microscope (Leica, Germany) using excitation at 405nm for DAPI and 488nm for GFP and recording the emission from 430 to 490 nm and from 505 to 515 nm respectively for DAPI and GFP. Single-focusing-plane images of 512x512 pixels were recorded with a scan speed of 400 Hz - - using 20x and 63x glycerol-immersion objectives.

2.6 GUS staining

For GUS staining, tissues were harvested, and transferred to GUS solution (50 mM Na-phosphate pH 7, 10 mM EDTA, 0.1% v/v Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 100 mg/mL chloramphenicol and 1.5 mg/mL 5-bromo-4-chloro-3-indoxyl-b-Dglucuronic acid, cyclohexylammonium salt (X-gluc, Biosynth AG, Staad, Switzerland)). Tissues were vacuum-infiltrated for 5 minutes, and subsequently left in the staining solution for 24 hours at 37ºC. For GUS staining in developing seeds, siliques were opened and fixed for 20 min at –20˚C in

90% acetone followed with 2 washes in GUS buffer without X-gluc prior to the staining procedure.

2.7 Plasmids and Cloning

The pPM6 vector has a pCambia 3300 (McElroy et al., 1995) backbone providing kanamycin resistance to bacteria and BASTA resistance to plants. To provide negative selectability to plants, the EcoRI/HindIII fragment of pNE3 vector (Stougaard, 1993) was inserted between EcoRI and HindIII sites of pCambia3300. To be able to introduce a rescuing gene into this construct we inserted a Gateway recombination cassette into the HindIII site of pPM2. The cassette was derived as an XhoI/SpeI blunt-ended fragment of pMDC15Ci (Dr. Mark Curtis, unpublished). The rescuing sequence of the *At2g37560* gene (*AtORC2*) was amplified from *Arabidopsis* genomic DNA using oligonucleotides ORC2gsGAT and ORC2gaGAT and subsequently introduced into pDONR207 (Invitrogen corp.) vector via Gateway cloning. Recombination between this vector and pPM2 produced the pPM5 vector, which thus contains the pCambia3300 backbone, the *CodA* gene controlled by CaMV 35S promoter, and the genomic sequence of the *AtORC2* gene.

The pPM4i vector has a pHellsgate8 (Helliwell and Waterhouse, 2003) backbone providing spectinomycin resistance to bacteria and kanamycin resistance to plants. Also, the sequence contains two Gateway recombination cassettes in inverse orientation separated by an intron. If transcribed, the inverted repeat forms an RNA hairpin, which can silence the expression of the gene with corresponding sequence. In order to make the expression of the hairpin inducible we exchanged the CaMV 35S promoter controlling the hairpin expression in pHellsgate8 with the minimal 35S promoter/lexA binding sites (35S/lexA). The 35S/lexA promoter was PCR-amplified from the pER8 vector (Zuo et al., 2000) using oligonucleotides LexA1d and lexA1r. To exchange it with the 35S promoter we first amplified the fragment of pHellsgate8 containing the first recombination cassette and a part of the intron with oligonucleotides PH1r and pmPH2d. The pmPH2d primer contained additional AvrII and SacI restriction sites on its 5' end, so that we could exchange the KpnI|SacI fragment of the PCR product with corresponding fragment of pHellsgate8, which produced the pHellsgate vector lacking the 35S promoter. Next, we introduced the

35S/lexA promoter between the AvrII and SacI sites of this vector, which resulted in formation of the pPM4i vector.

The Gateway cassettes in pPM4i were exchanged with a fragment of the AtORC2 gene via Gateway cloning producing the pPM4i-O2 vector. The ORC2 fragment was PCR-amplified using oligonucleotides ORCiFg and ORCiRg.

The pPM12 vector has the pMDC110 (Curtis and Grossniklaus, 2003) backbone providing kanamycin resistance to bacteria and hygromycin resistance to plants. Also, pMDC110 carries the sequence of mGFP6 (Haseloff, 1999) downstream of the Gateway recombination cassette to allow the production of a fusion protein that carries the GFP on its C-terminus. It was not possible to introduce the ORC2 sequence into the pMDC110 by Gateway cloning, so I excised the Gateway cassette between AscI and PacI sites and replaced it with the AtORC2 genomic sequence amplified by oligonucleotides Orc2gaAsc and Orc2gsPac.

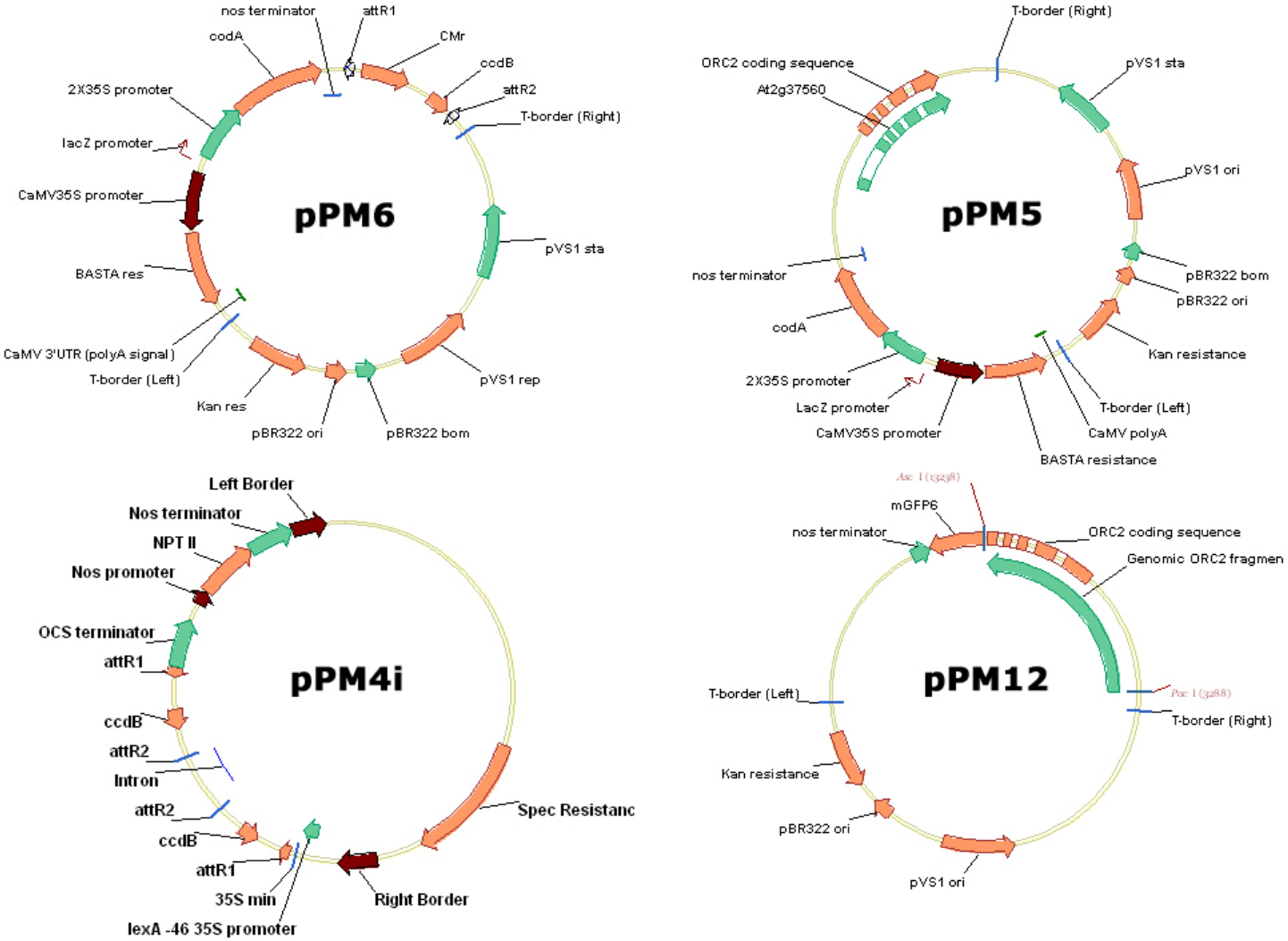


Figure 2-1. Maps of vectors used

2.8 Nuclei preparation, Fluorescent In Situ Hybridization (FISH) and ploidy analysis

FISH was performed as described in (Fransz et al., 1998) with modifications. Siliques were opened with insulin needles (Becton Dickinson, Franklin Lakes, NJ). The seeds were taken out and fixed in 1% formaldehyde in Tris buffer (10 mM Tris, 10 mM EDTA, 100 mM TritonX-100, pH 7.5) for 1h on ice, washed twice with Tris buffer, and mechanically disrupted in nuclei isolation buffer (10 mM Tris-HCl, pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1.0 mM spermine, 0.1% mercaptoethanol). The nuclear suspension was placed onto microscopic slides, air-dried and fixed with ethanol:acetic acid (3:1) solution for 2 minutes, and baked for 30 min at 60°C. Slides were pre-fixed in 1% paraformaldehyde, washed in PBS, treated with 0.3% pectinase-cellulase-cytohelicase mix in citrate buffer 10 mM sodium citrate pH 4.5 for 10 min at 37°C, washed with 2xSSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate), then treated with 50µg/ml pepsin in 0.01 N HCl at 37°C for 10min, washed in 2 x SSC and treated with 100µg/ml RNase A in 2xSSC for 30 min at 37°C. Centromeric probe was obtained using the biotin nick translation kit (Roche) with a plasmid (pAl1) ((Martinez-Zapater, 1986) containing the *Arabidopsis* centromeric repeat. Digoxigenin-containing probes were obtained using the DIG-labelling kit (Roche). Nucleolar organizer probe was obtained using the plasmid pTA71 that contained 45S rDNA (Barker et al., 1988). Euchromatic probes were made using BAC clones F10O3 (chromosome 1, bottom arm) and T18B16 (chromosome 4, top arm). For each slide, 3 µl of each probe was ethanol-precipitated and resuspended in 10ul formamide (Sigma), then mixed with 10 µl 2xDS solution (20% dextrane sulphate in 0.05 M sodium phosphate buffer pH 7.0, 0.3 M NaCl and 0.03 M sodium citrate). Probe mix was denatured for 15 min at 75°C, chilled on ice and then applied onto the slide. Slide and probe were denatured for 2 min at 80°C, and then transferred to a moist chamber preheated to 55°C. Hybridisation was performed at 37°C for ~12 hours. The biotin-labeled probe was detected with avidin conjugated with Texas Red (Vector Laboratories), followed with a biotinylated goat-anti-avidin antibody (Vector Laboratoties) and once again Texas Red-avidin. DIG-labelled probes were detected with mouse-anti-digoxygenin antibody (Roche) followed with goat-anti-mouse antibody conjugated with Alexa488 (Molecular Probes, Invitrogen

Corp.) fluorescent stain. DNA was counterstained with 4',6-Diamidino-2-phenylindol (DAPI) in Vectashield mounting media (Vector Laboratories, Inc., Burlingame, CA 94010). Nuclei were inspected with a Zeiss Axioplan epifluorescence microscope and images were recorded with an Apogee AltaU32 camera. Images were merged and processed using Adobe Photoshop 5.5 software.

For ploidy analysis, nuclei were prepared as described above and stained with DAPI. Images were recorded as above and fluorescence intensity was measured for individual nuclei using ImageJ software v1.33u (Abramoff et al., 2004).

3. RESULTS

The focus of this thesis work was the second subunit of the *Arabidopsis thaliana* Origin Recognition Complex. My initial interest in this protein originated from the peculiar phenotype exhibited by the *orc2-1* mutant (line ET3753) identified in the screen for semisterility in a collection of enhancer detector *Ds* transposants (Moore et al., 1997). This mutation is zygotically lethal. Mutant embryos arrest at the preglobular stage of development, and are disorganized. The endosperm in mutant seeds proceeds only through 2-3 rounds of division and the nuclei are dramatically enlarged compared to wild-type nuclei and have a giant nucleolus. Appearance of giant nuclei in a mutant for a protein with a presumably replicative function was surprising. Finding an explanation for this discrepancy was the main motivation to work on this project.

To find out more about the role of AtORC2 in seed development, I performed a more detailed phenotypic analysis of the *orc2-1* mutant (section 3.1) and also characterized two additional mutant alleles (section 3.1.2).

3.1 Phenotypic analysis of the orc2-1 mutant

3.1.1. orc2-1 embryos develop slowly and are abnormally patterned.

Siliques of plants heterozygous for the *orc2-1* mutation were cleared and the developing seeds observed with Nomarski optics. Mutant seeds were compared with their phenotypically wild-type (*orc2-1*/*ORC2* and *ORC2/ORC2*) siblings to characterize their development at different times after fertilization. *Orc2-1* mutant embryos do not develop at normal rate of progression and then stop before abortion; instead their whole development was substantially slowed down (Figure 3-1). Also, the patterning of *orc2-1* embryos was aberrant (Figure 3-2). First, there was an increased level of asynchrony in early embryo divisions (Table 3-1). Asynchrony was measured as the proportion of embryos with an intermediate

number of cells in the embryo proper (3 or 5-7 cells for transitions from bicellular to quartet and from quartet to octant stage, respectively). The increased asynchrony was also observed in *orc2* mutant embryos expressing *CycB1:*1::GUS:DB reporter construct (Figure 3-3.). This construct expresses GUS in G2 phase of the cell cycle and in M phase GUS is degraded due to the presence of the Destruction Box sequence. Thus, this construct can be used to monitor the progression of the cell cycle. Second, already starting from the very first divisions of the apical cell, cell division planes were not characteristic for the stage of embryo development. The occurrence of such abnormal division planes increased as the mutant embryos developed (Table 3-2).

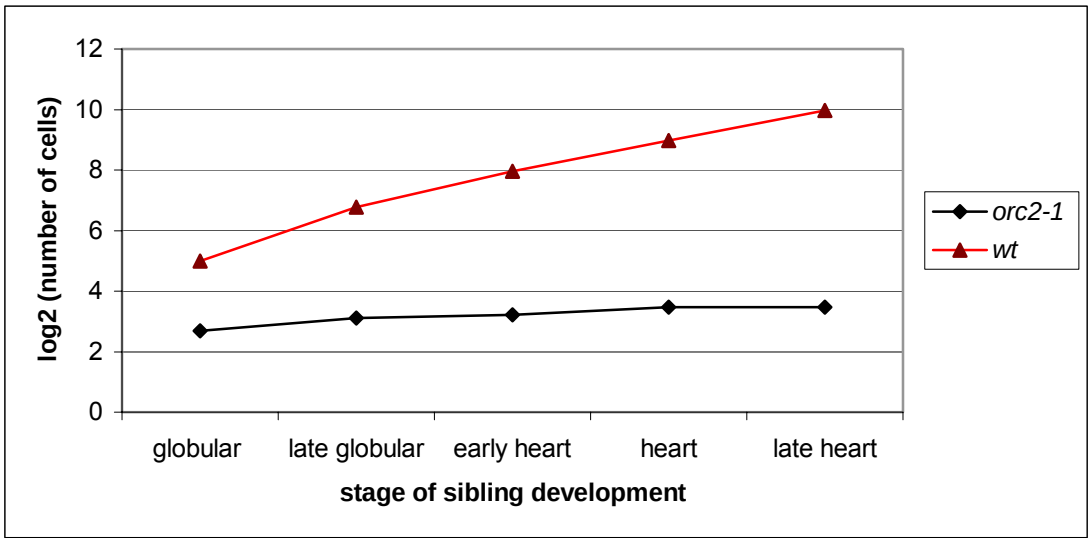


Figure 3-1. Embryo proliferation dynamics in wild-type and in the *orc2-1* mutant. Cell number in wild-type and *orc2-1* embryos at different stages of development. Mutant embryos in all lines develop slower than their wild-type siblings and abort before reaching globular stage.

	Embryos with asynchronously divided cells, %		Embryos with abnormal division planes, %	
Embryo-proper stage	*orc2-1*	wild-type	*orc2-1*	wild-type
2-4 cells	11.3, n=53	3.8, n=52	34, n=53	0
4-8 cells	32.8, n=61	16.4, n=43	44.5, n=63	0
Combined	22,8, n=114*	9.5, n=95*		

Table 3-1. Abnormal embryo divisions in the seeds of *orc2/ORC2* plants. Embryos with asynchronous divisions were those with an intermediate number of cells in the embryo proper (3 or 5-7 cf. the series of 2, 4, 8, 16 characteristic of normal development), or cells divided in a non-characteristic plane. In order to detect occurrence of abnormal division planes in wild-type, 4 siliques (approx 200 seeds) were inspected. For fields marked with asterisk Fisher's exact test was applied and the difference between mutant and wild type was found to be significant with $p<0.01$.

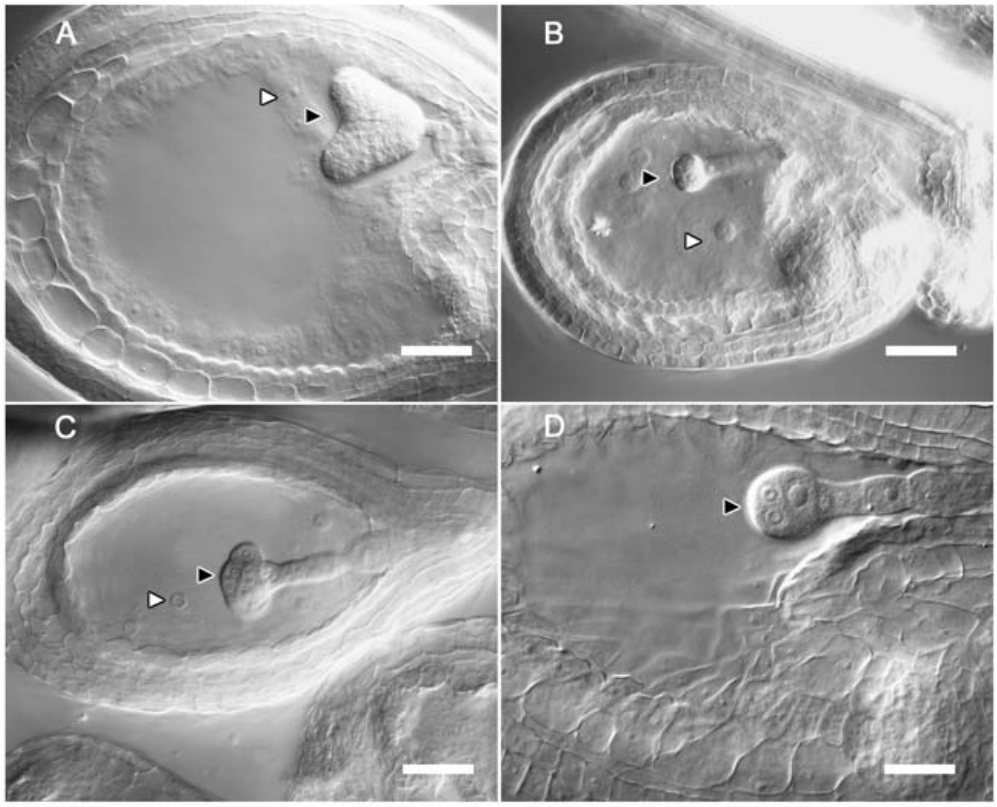


Figure 3-2. Seed development in the *orc2-1* mutant. At the time wild-type embryos reach heart stage (**A**), *orc2-1* mutant seeds contain a preglobular embryo and a small number of endosperm nuclei, which are enlarged. As mutant embryos develop, they acquire abnormal shape (**C**). However, abnormal divisions start to happen as early as the first division of the apical cell in a 2-cell embryo. **D:** a four-cell *orc2-1* embryo, in which the first division of the apical cell occurred in the plane perpendicular to the embryo axis. Black arrowheads: embryos, white arrowheads: endosperm nuclei. Bar in A to C: 100µm, in D: 50µm.

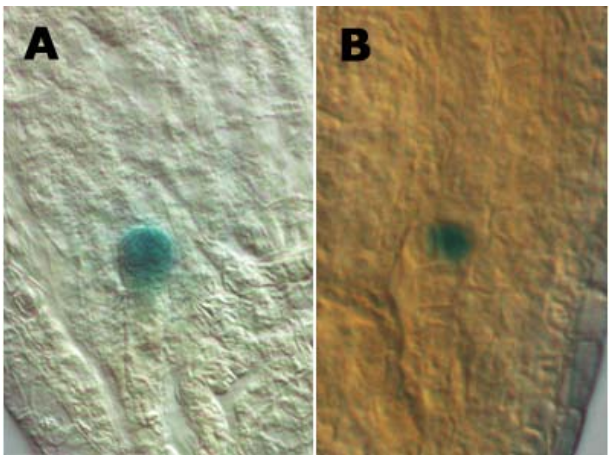


Figure 3-3. GUS staining of seeds carrying an insertion of *CycB1;*1::GUS construct in wild-type and *orc2-1* background. A. Wild-type 4-cell stage embryo. All cells in embryo proper are stained. **B.** 2-cell *orc2-1* mutant embryo. Only one cell in the embryo proper expresses the marker.

3.1.2 Mutant alleles of ORC2.

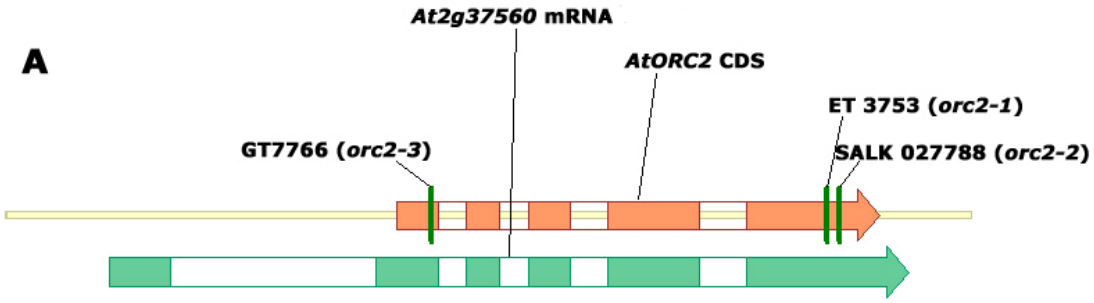
To learn whether the *orc2-1* phenotype is unique to this allele, or common among *ORC2* mutants, we obtained two other plant lines carrying an insertion in the *ORC2* gene: SALK_027788 (*orc2-2*) and GT7766 (*orc2-3*) (the *AtORC2* gene model and the positions of insertions are depicted in Figure 3-2, A). Both mutations were found to be zygotically lethal; inspection of opened siliques revealed that ¼ of seeds abort. When crossed to each other and to *orc2-1*, the rate of seed abortion was found to be the same, demonstrating that the mutants are indeed allelic.

To investigate possible differences of phenotypes in different alleles, I performed microscopical analysis of cleared siliques of heterozygous plants of each line. The results are summarized in Figure 3-4. In all three lines homozygous embryos stopped their development before reaching the globular stage of development and all contained enlarged endosperm nuclei with the exception of the *orc2-3* line, where a wide range of number and size of endosperm nuclei was observed (for details see section **5-2.**). In all lines, mutant embryos developed slower than siblings carrying a wild-type copy of the *ORC2* gene (Figure 3-4), and had cell division planes non-typical for their developmental stage. However, in different lines such abnormal divisions occurred at different frequencies among the three alleles, and embryos stopped their development at different stages (Figure 3-4).

Differences in embryo development were neglectable between *orc2-1* and *orc2-2* alleles, but the endosperm proceeded through fewer rounds of nuclear divisions in the *orc2-2* mutant. Also, the size of endosperm nuclei in this line seemed to be

smaller. The *orc2-3* line is different from *orc2-1* and *orc2-2*. The embryo stopped its development earlier, rarely reaching the quadrant stage. A great variability in the endosperm nuclei number was observed between individual seeds. Often their number nearly identical to that of the wild type (Figure 3-5, F; Supplementary Chart 1). This finding was surprising: the *Ds* element insertion causing the *orc2-3* mutation is located at the very beginning of the *AtORC2* gene (Figure 3-4, A.), and I expected to see the most severe phenotype in this line.

A



B

	Average cell number			
stage	*orc2-1*	*orc2-2*	*orc2-3*	wt
globular	3.5, n=33	2, n=74	1.3, n=10	32
late globular	4.8, n=9	3.1, n=59	1.8, n=17	110
early heart	5.2, n=14	4.2, n=18	2, n=11	250
heart	6, n=16	4.4, n=19	2, n=15	500
late heart	6.2, n=14	5, n=15	2.3, n=18	1000

C

stage	*orc2-1*	*orc2-2*	*orc2-3*	wt
globular	3.3	2.1	26.2	90
late globular	4.3	2.0	20.8	150
early heart	3.6	1.5	28.2	200
heart	3.7	1.3	32.2	Over 200
late heart	3.1	1.6	34.1	Over 200

Figure 3-4. Proliferation of *orc2* mutant embryos and endosperm compared to wild-type. A. *AtORC2* gene model. Positions of insertions are indicated. **B.** Average cell number in embryo propers of mutant and wild-type embryos at different stages of development. Values for wild-type are taken from published data (Jenik et al., 2005). **C.** Average number of endosperm nuclei observed in seeds of *orc2* mutants at different time points. Sample sizes are same as in A. Numbers for wild-type are taken from published data (Boisnard-Lorig et al., 2001).

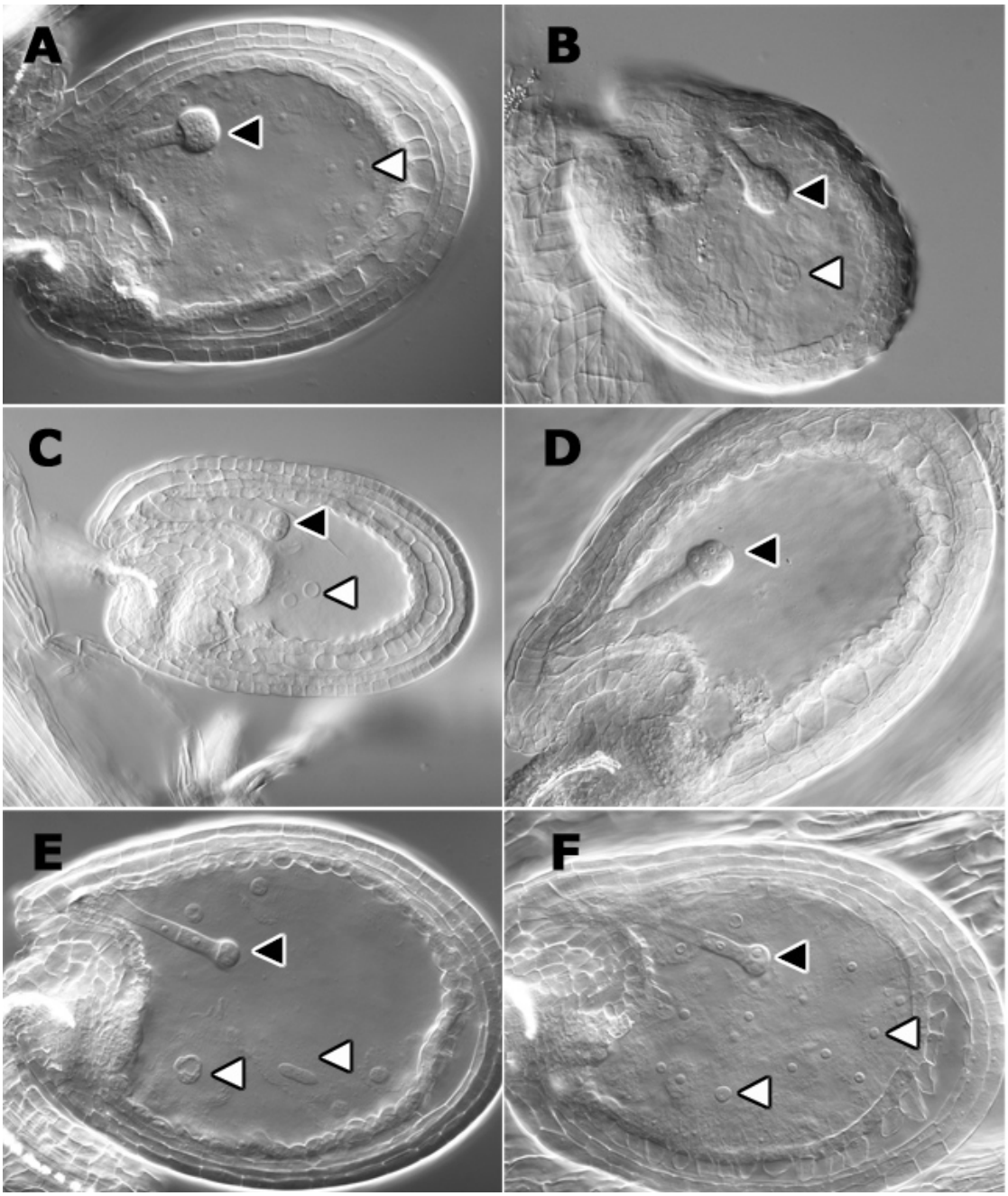


Figure 3-5. DIC microscopy images of cleared seeds of different *orc2* alleles. White arrowheads point at endosperm nuclei, black arrowheads point at embryos **A.** Wild-type seed with an embryo of globular stage. **B.** *orc2-1* seed at a terminal stage. Large globular embryo of abnormal shape is visible, as well as a giant endosperm nucleus. **C, D.** Seeds of the *orc2-2* mutant. **E, F.** Seeds of the *orc2-3* mutant. Variation between individual seeds in number and sizes of endosperm nuclei was observed.

One of the explanations for the unexpectedly weak endosperm phenotype in *orc2-3* mutants is that a small amount of AtORC2 protein is produced in the endosperm that allows it to proliferate further than in the other two alleles. I see two possibilities how this could happen. First, a truncated/modified version of the protein could be translated from a transcript that contains both the AtORC2 sequence and the sequence of the inserted *Ds* element. Secondly, it has been shown that *Ds*-elements can be post-transcriptionally spliced out of the nascent transcript, leaving a footprint in the resulting mRNA (Wessler, 1991). Detection of an *ORC2* mRNA that contained either a complete *Ds*-element, or a footprint at the insertion site would constitute evidence for the existence of a small amount of the functional ORC2 protein in the endosperm of *orc2-3* mutant seeds.

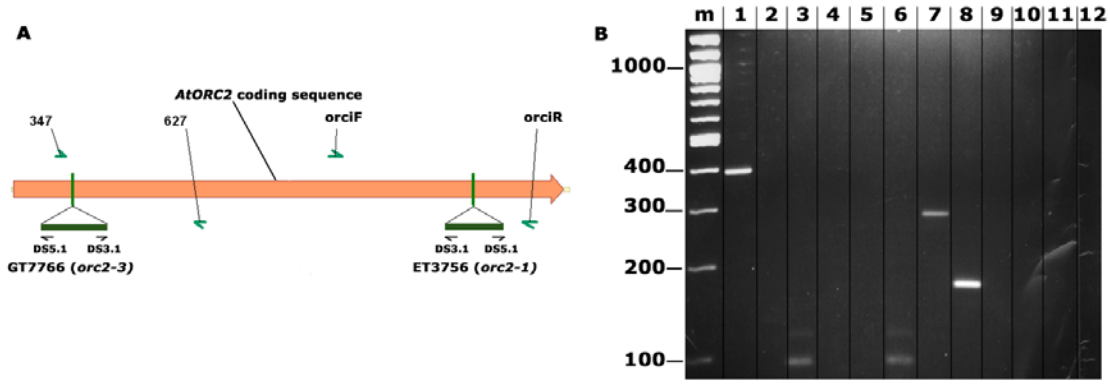


Figure 3-6. *AtORC2* coding sequence and detection of aberrant transcripts in *orc2-1* and *orc2-3* mutants. **A.** *AtORC2* coding sequence showing the two transposon insertion positions, as well as positions of oligonucleotides used for RNA analysis in mutant lines. **B.** RT-PCR analysis of *ORC2* mRNA in *orc2-1* and *orc2-3* mutant lines. 1-6: mRNA from *orc2-1* seeds. 7-12: mRNA from orc2-3 seeds. Oligonucleotide pairs used for amplification: 1,3: ORCiF/ORCiR (fragment size expected from wt mRNA: 392b); 2,4: ORCiF/DS3.1, 3,6:ORCiR/DS5.1; 7,10: 347/627 (fragment size expected from wt mRNA: 281b); 8,11: 347/DS5.1; 9,12: 627/DS3.1. Lanes 4-6 and 10-12 serve as controls for the absence of genomic DNA contamination, as no reverse transcriptase was added to these RNA samples.

Detection of an mRNA species solely in mutant endosperm would require getting rid of the maternal tissues, which is laborious and requires complex equipment (such as laser-assisted capturing microscope (Emmert-Buck et al., 1996; Bonner et al., 1997)). Instead, I assayed for an mRNA species that is

different from wild-type *ORC2* transcript by RT-PCR. The positions of the *Ds* insertions into the *ORC2* gene in *orc2-1* and *orc2-3* lines, as well as positions of oligonucleotides used in the analysis are shown in Figure 3-6, A. Figure 3-6, B. represents the results of these experiments. No *ORC2* mRNA species of a size substantially different from wild-type was detected either in seeds of the *orc2-3* or the *orc2-1* line. Notably, a polyadenylated transcript that contains both the 5' end of *ORC2* mRNA and at least a fragment of the *Ds* element was detected in the *orc2-3* line (Figure 3-6, B., lane 8). However, because the *Ds* insertion in this line is located at the very beginning of the ORC2 coding sequence, the existence of a transcript containing only the 5' fragment of the ORC2 mRNA cannot explain the increased proliferation of endosperm in *orc2-3* seeds.

3.1.3 Giant endosperm nuclei reach abnormally high ploidy levels.

The endosperm nuclei in the mutant seeds of the *orc2-1* line are bigger than in the *orc2-3* line (Figure 3-5), and their number is greater than in the *orc2-2* line (Figure 3-4). A detailed analysis of giant endosperm nuclei was therefore performed in the *orc2-1* line. To get insights into the structure of the giant endosperm nuclei of the *orc2-1* mutant, I extracted the endosperm from mutant seeds, stained it with DAPI and inspected it with an epifluorescence microscope. Confirming the observations with DIC microscopy, mutant endosperm nuclei appeared much larger than in wild-type and heterozygous seeds, ranging between 15-70 µm as compared to 9-12 µm of wild-type peripheral endosperm nuclei (PEN). Mutant nuclei were also readily distinguishable from Chalazal Endosperm Nuclei (CZE), which had a size of 15-30 µm, but had a much smaller nucleolus, and well-defined chromocenters (Figure 3-7, A-C). In contrast, chromatin of mutant endosperm nuclei appeared diffuse, with no prominent chromocentres. The nuclei often contained multiple enlarged nucleoli.

To learn whether the increased physical size of mutant nuclei is a consequence of increased DNA content, or of decondensation of the chromatin, I measured total fluorescence from wild-type and mutant nuclei, and found that the ploidy level of *orc2-1* mutant endosperm nuclei can reach up to 170C, as compared to the maximum of 21C observed in wild-type endosperm nuclei (Figure 3-7, D). Clearly,

endosperm nuclei in the *orc2-1* mutant undergo extensive endoreplication after arresting division, which is not intuitively consistent with a block of DNA replication that might be expected after loss of *AtORC2*. DAPI staining also showed that the giant nuclei had no distinct chromocentres, indicating that heterochromatin formation might be aberrant.

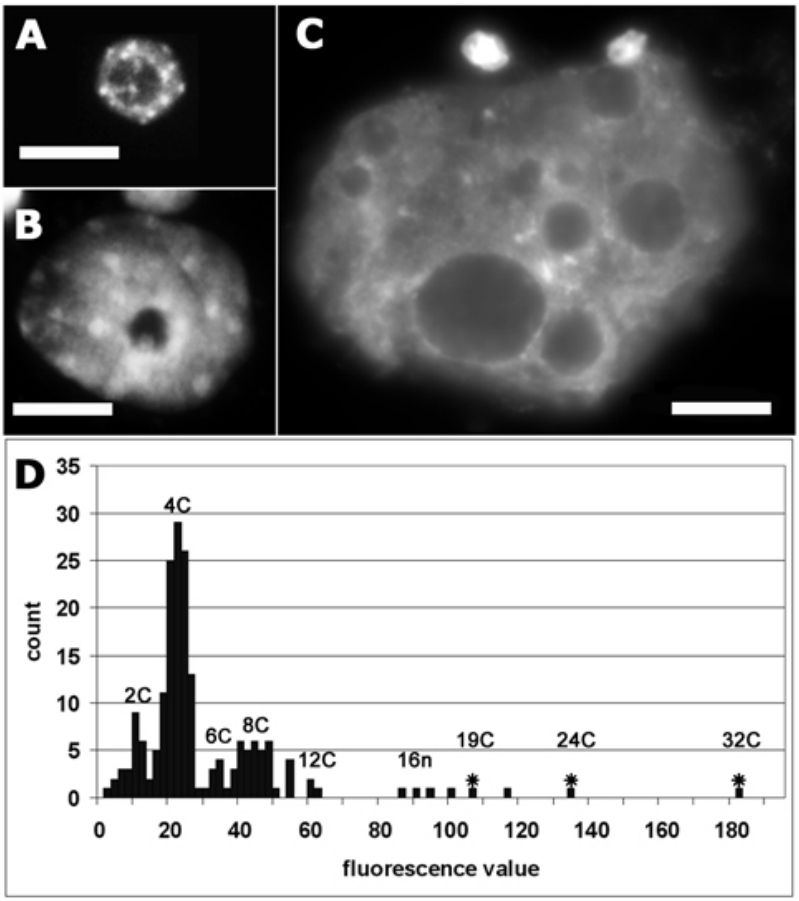


Figure 3-7. Endosperm nuclei of the *orc2-1* mutant reach abnormally high ploidy levels. DAPI staining of endosperm nuclei from normal (**A:** PEN nucleus, **B:** CZE nucleus) and *orc2* mutant (**C**) seeds. Bar: 10 µm. **D.** Combined ploidy profile of wild-type and *orc2* mutant (asterisks) squashed seeds.

3.1.4 Heterochromatin structure is affected in mutant endosperm nuclei.

In addition to its function in the initiation of DNA replication, the ORC2 protein has also been shown to be involved in silencing and heterochromatin formation. In eukaryotes, heterochromatin is usually formed at loci harboring repetitive sequences. The amount of such sequences in the genome of *A. thaliana* is relatively small (Meyerowitz, 1994) compared to other eukaryotes. Loci with

repeats that become heterochromatized are centromeres, mainly consisting of the 180bp-long centromeric repeat (Brandes et al., 1997), and the two nucleolar organizers, located on chromosomes 2 and 4, that comprise the 45S ribosomal DNA (rDNA) genes (Bauwens et al., 1991; Haberer et al., 1996; Fransz et al., 2003). The centromere is a major structural component of eukaryotic chromosomes, being the place of kinetochore assembly, a structure necessary for chromosome segregation during mitosis. Disruption of the centromeric structure might hamper chromosome segregation during mitosis, and hence, might lead to the formation of a giant nucleus

To test whether the heterochromatin is affected in *orc2* mutant PEN, I performed FISH analysis of mutant endosperm nuclei with probes corresponding to *Arabidopsis* centromeric repeats. Further, to test whether other heterochromatic regions are also affected by the *orc2* mutation, and to learn more about the state of endoreplicated chromosomes in the mutant PEN, we performed hybridizations to nucleolar organizers (NORs) and a number of selected euchromatic regions. In this manuscript, I present FISH results for two BAC clones corresponding to two different euchromatic loci on chromosomes 1 and 4. Hybridizations with BAC clones corresponding to other euchromatic regions revealed similar patterns.

In wild-type PEN, centromeric repeats are organized in compact structures that coincide with bright spots in DAPI-stained nuclei (Figure 3-8 A, 1-3). In the *orc2-1* mutant nuclei, the normally heterochromatic centromeric repeats often appear disorganized and dispersed within the nucleus, with “bridges” of centromeric DNA connecting spots of higher intensity (Figure 3-8 A, 4-6). In wild-type endosperm nuclei the number of distinguishable spots of centromeric DNA varied between 9 and 14, with an average of 11. The number of distinguishable centromeric spots in mutant nuclei varied, and based on this feature, the observed mutant nuclei fell into two classes. In nuclei of the first class, loci harboring centromeric repeats were separated from each other, and their number often exceeded 40 (Figure 3-8, C). The second class consisted of nuclei in which no distinguishable spots of centromeric DNA could be identified, and the fluorescent signal from the centromeric repeat probe was distributed all over the mutant nucleus, and spots were interconnected with each other (Figure 3-8 A, 4-6).

In a wild-type PEN hybridized to 45S rDNA probe, two nucleolar organizer regions (NOR) were usually observed that each had two characteristic domains. First, decondensed regions that protruded into the nucleolus, and second, condensed spots that lay at the periphery of the nucleolus and could be seen as spots of a higher intensity in DAPI-stained nuclei (figure 3-8 B, 1-3). In the mutant nuclei, I did not observe such spots, and the NORs were highly decondensed and occupied large regions around and within the nucleoli. Although the increased DNA content of the mutant nuclei in part accounts for the increase in the area occupied by NORs, the absence of well-defined heterochromatic spots confirms that AtORC2 functions in heterochromatin formation. In accordance with the increased amount of DNA in mutant endosperm nuclei, I observed larger numbers of FISH signals corresponding to each of two euchromatic BAC clones than in wild-type PEN. These signals did not form clusters in mutant nuclei, which was also similar to wild-type PEN. Hence, I observed no evidence for any effect of the *orc2-1* mutation on the structure of euchromatin.

The results show that the *Arabidopsis orc2-1* mutation leads to the formation of giant endosperm nuclei with dramatically increased DNA content, and disturbed heterochromatin structure. This shows that the function of the ORC2 protein in chromosome condensation is also conserved in plants. In *Drosophila*, the ORC2 protein has been shown to have a function in the condensation of mitotic chromosomes (Pflumm MF, 2001). A failure to condense chromosomes properly at the onset of mitosis could theoretically hamper chromosome segregation and block mitosis. If the nucleus initiated new rounds of DNA replication without mitosis it would lead to the formation of polyploid nuclei I observed in the *orc2-1* mutant endosperm. In contrast to the *titan* mutants (Liu and Meinke, 1998) I never observed *orc2-1* endosperm nuclei undergoing mitosis. This would be consistent with a block in mitosis, but also meant that it was not possible to assess whether *orc2* mutant endosperm nuclei have a defect in mitotic chromosome condensation.

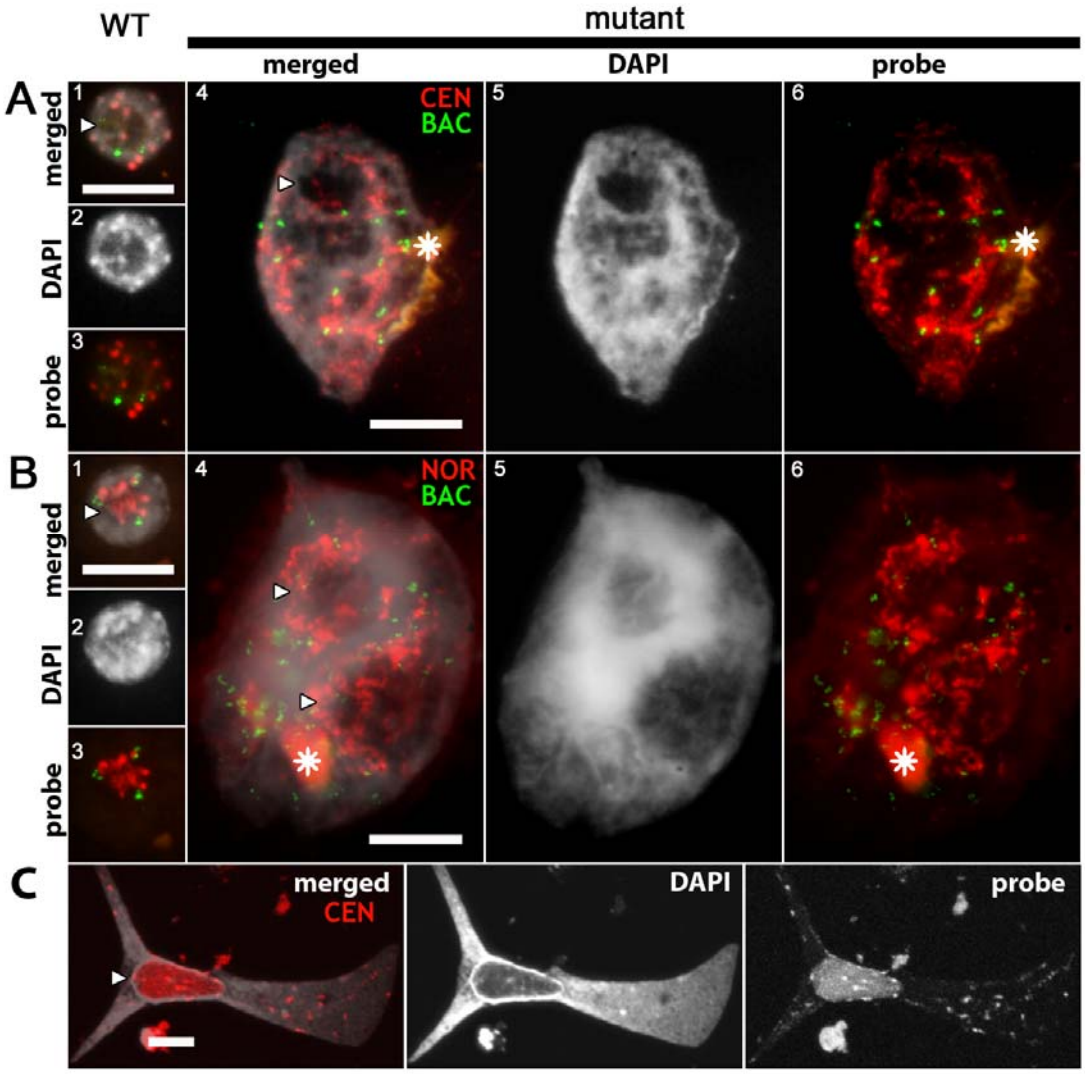


Figure 3-8. FISH on endosperm nuclei of wild-type and *orc2-1* mutant seeds. A. Centromeric repeat probe detected as red signal and a euchromatic region of chromosome1 (BAC clone F10O3) as green. B. NOR detected as red signal and a euchromatic region of chromosome 4 (BAC clone T18B16) as green. C. Centromeric repeat probe detected as red signal. DAPI staining in gray, bar: 10 µm. Arrowheads point to nucleoli, asterisks mark regions of unspecific hybridization.

3.2 ORC2 protein localizes to DNA in interphase nuclei and leaves it during mitosis

3.2.1 The *AtORC2:GFP* construct

The occurrence of abnormal division planes observed in *orc2* mutant embryos suggested that the AtORC2 protein has a possible function in cell polarity determination and positioning of the cell plate. The notion that the ORC2 protein has a role in mitosis in human cells (Prasanth et al., 2004) supports this hypothesis. To investigate this possibility, I generated a construct, pPM12, in which the coding sequence of the *ORC2* gene was fused in frame with the sequence of Green Fluorescent Protein, GFP (Figure 3-9). The expression of the fusion construct was controlled by the native ORC2 promoter. I introduced the pPM12 construct into *orc2-1* heterozygous plants. Among 12 T1 plants tested, I identified one (PM94), all of whose viable progeny were carrying both the *orc2-1* mutation, and the pPM12 construct. Inspection of opened siliques of this plant revealed that ¼ of the seeds aborted. Together, these results show that the PM94 line was homozygous for the *orc2-1* mutation and hemizygous for the pPM12 insertion segregating as a single locus, unlinked to *orc2*. Overall, these plants were phenotypically indistinguishable from the parent line. This demonstrates that the ORC2:GFP fusion protein expressed from the pPM12 vector is able to restore the function of the ORC2 protein.

3.2.2 Intracellular localization of ORC2:GFP fusion protein through the cell cycle

In the plants of PM94 line, GFP expression was detected in the female gametophyte (Figure 3-11, A-C), male gametophyte (pollen, Figure 3-12) and developing seeds (Figure 3-11, D), thus confirming the data presented in (Collinge et al., 2004) obtained by *in situ* hybridization. In addition, elevated levels of GFP fluorescence were found in both shoot (not shown) and root apical meristems (Figure 3-10).

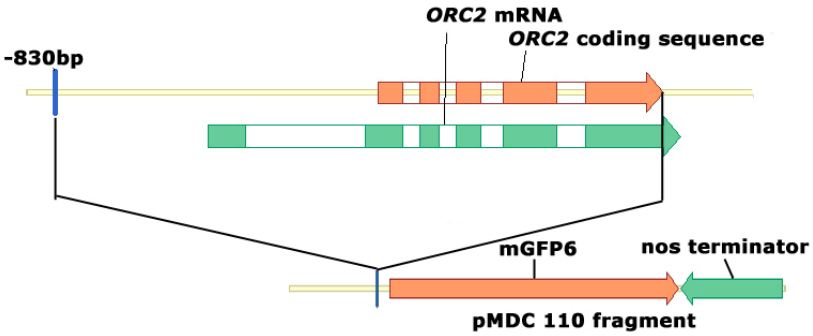


Figure 3-9. Scheme of the construct expressing ORC2:GFP fusion protein (a fragment of pPM12 vector). A fragment of the *AtORC2* gene (from the position –830bp from transcription start to the last coding triplet) was inserted in frame upstream of mGFP6 sequence in the pMDC110 vector to create the pPM12 vector. Thus, the expression of the fusion protein was controlled by the native *AtORC2* promoter element and nos terminator.

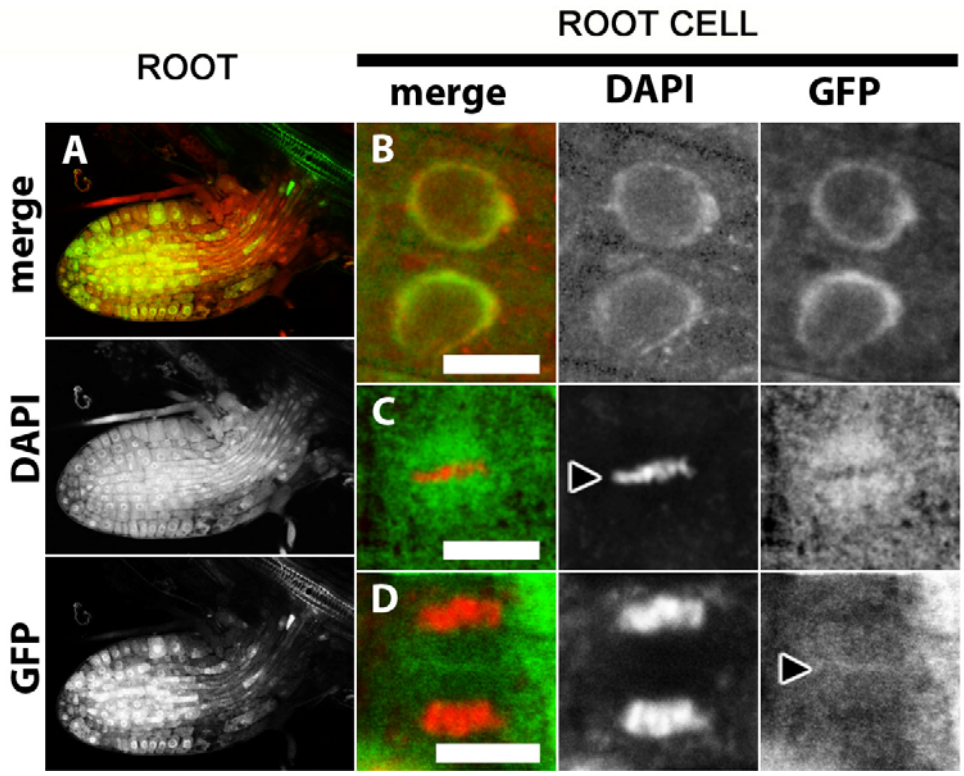


Figure 3-10. CLSM images of seedlings expressing AtORC2 fused to GFP. A. Expression of AtORC2 in root meristem. B-D. Subcellular distribution of AtORC2 compared with DAPI stained DNA during the cell division cycle in root meristem cells; B, interphase, C, metaphase, with arrowhead showing the chromosomes at the metaphase plate, D, telophase, with arrowhead showing the developing cell plate. Bar: 10μm

Observations of the root meristem cells at the intracellular level revealed that during interphase ORC2:GFP locates predominantly to the nucleus, co-localizing with DNA (Figure 3-10, B). Since AtORC2-GFP levels were only high in the cell division zone of the meristem, it was not possible to look at its subnuclear distribution in non-dividing cells with pronounced chromocentres. However, in cells expressing detectable levels of AtORC2:GFP, the distribution of AtORC2 on the DNA was uniform; I saw no evidence for accumulation at either heterochromatic or other foci. A substantial pool of AtORC2-GFP was seen in the cytoplasm (Figure 3-10 and Figure 3-11), which may reflect a role for *AtORC2* unconnected with genome replication and organization, or be an artefact of the fusion protein. Accumulation of AtORC2-GFP in the cytoplasm due to the relatively high expression levels in the PM94 line seems unlikely as independent AtORC2-GFP transformants with lower expression levels had similar distribution patterns.

During mitosis, from metaphase and through telophase, the fusion protein leaves the DNA, and the signal resembles the structure of the mitotic spindle, suggesting association of the AtORC2 protein with the microtubule apparatus (Figure 3-10, C). The presumptive association with microtubules was preserved up to late telophase: the GFP signal was observed at the place where the new cell plate is formed (Figure 3-10, D). This distribution of the ORC2 protein is similar to observations made in mammalian cells, where ORC2 and ORC6 proteins were observed not only associated with DNA, but also with the microtubule apparatus (Prasanth et al., 2002; Chesnokov et al., 2003; Prasanth et al., 2004). Our observations fit well with these data and bring further evidence for ORC subunits also being involved in cellular processes outside S-phase.

3.2.3 AtORC2:GFP localization in gametophytes

Many aspects of the cell cycle differ between somatic and germline cells. In *Xenopus*, the affinity of ORC to the chromatin was different in somatic and egg cell extracts (DePamphilis, 2005). To learn about the ORC2 localization in haploid tissues, we inspected the distribution of ORC2:GFP in *Arabidopsis* gametophytes. In the mature female gametophyte GFP signal is also predominantly nuclear, and especially strong in the central cell and the egg cell nuclei (Figure 3-11, B-C). In

mature pollen, surprisingly, the signal from the ORC2:GFP construct is associated predominantly with the vegetative nucleus but is nearly undetectable in sperm nuclei (Figure 3-12). We observed the same distribution pattern also in growing pollen tubes, which is unexpected because sperm nuclei are in the S-phase of the cell cycle during the growth of the pollen tube (Friedman, 1999).

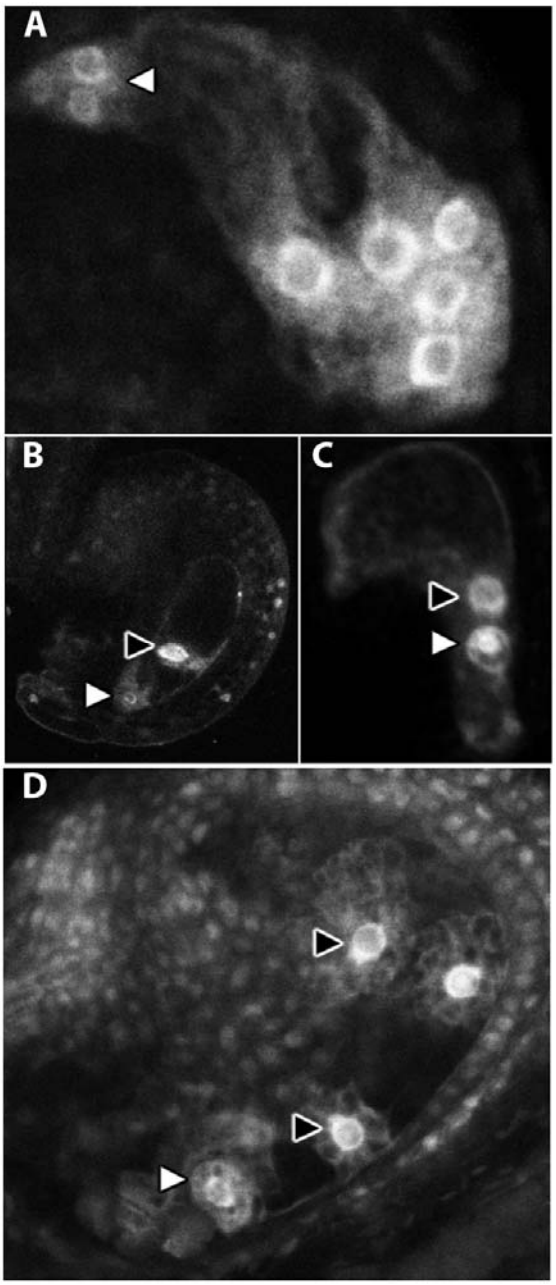


Figure 3-11. CLSM images of the ORC2:GFP fusion protein in developing female gametophyte and in early embryo sac. A: Immature female gametophyte. GFP signal observed in all 8 nuclei. White arrowhead points to antipodal nuclei. **B and C:** Mature female gametophyte. White arrowheads point to egg cell, black arrowheads point to central cell. **D:** Embryo sac shortly after fertilization. Endosperm has completed two rounds of divisions. White arrowhead points to the zygote, black arrowheads point to endosperm nuclei.

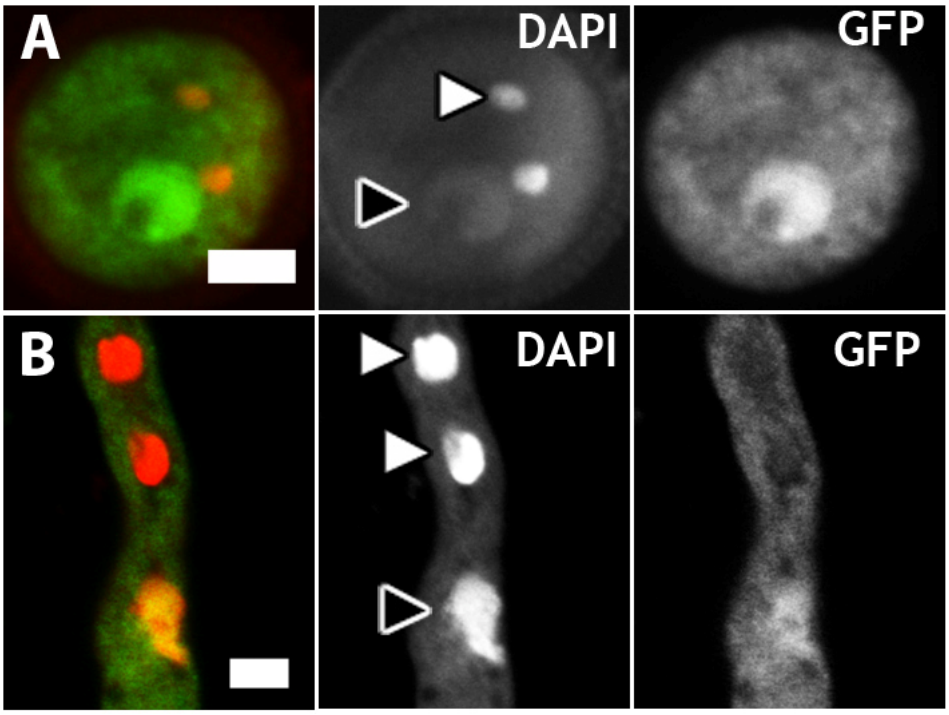


Figure 3-12. CLSM images of ORC2:GFP construct expression in pollen. **A:** Mature pollen grain. **B:** Growing pollen tube. White arrowheads point at sperm cells, black arrowheads point at the generative cell nucleus. Bar: 5µm

3.3 The suppressor screen.

Suppressor/enhancer screens have proved to be a powerful tool in dissecting pathways and determining members of protein complexes (Page and Grossniklaus, 2002). Although they can be performed in many ways, the common feature among them is that they are performed in a mutant background. That is, the ultimate goal of any suppressor/enhancer screen is to identify a second-site mutation that will alter (enhance or suppress) the phenotype of the background mutation.

The *orc2-1* phenotype can be partially rescued by another mutation, *medea* (Collinge et al., 2004). This discovery led to the assumption that a second-site mutation could exist that could rescue the phenotype caused by the *orc2-1* mutation. In order to find such a mutation, it was proposed to perform a screen for a mutant that is able to survive without a functional ORC2 protein. The screening strategy is outlined in the Figure 3-13.

The proposed screen must be performed in an *orc2/orc2* background. In order to recover such a plant, we needed to introduce a rescue construct into *orc2-1/ORC2* plants. The rescue construct had to be closely linked to a negatively selectable marker, so that mutagenized seeds carrying the rescuing copy of the *ORC2* gene would not be able to germinate. A suppressor mutant would be identified as a plant that can survive the negative selection. Such suppressor mutations may be recessive, and therefore selection must be performed in the M2 generation, where homozygotes for the suppressor first segregate. Thus, a plant that is able to grow and to survive the negative selection for the transgene in the M2 generation after the EMS mutagenesis, should contain either a damaged negatively selectable marker, or the mutation of interest.

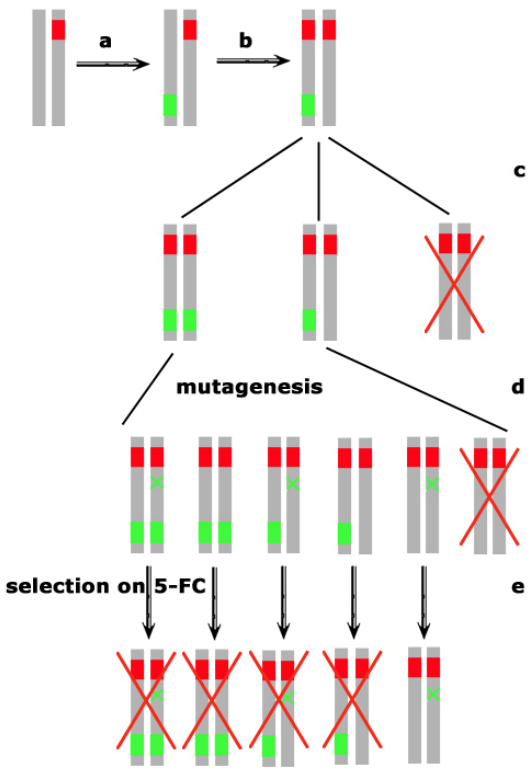


Figure 3-13. Scheme of the proposed screening strategy. Grey line represents haploid genome. Red box represents *orc2* mutation. Green box represents the construct containing a rescuing wild-type copy of the *ORC2* gene and the negatively selectable marker (CodA). Green cross represents the modifier/suppressor mutation that allows plants to survive in absence of the ORC2 protein. The rescuing construct is introduced into *orc2/ORC2* plants (**a**) and (**b**) a line homozygous for the mutation and hemizygous for the insertion is selected. The progeny of this line (**c**), which consists of plants homozygous for the orc2 mutation and homo- or hemizygous for the insertion, is mutagenized (d). In the presence of 5-FC only those plants that do not carry the negatively selectable marker are able to survive.

As a negatively selectable marker we selected the bacterial CodA gene, encoding cytosine deaminase. Cytosine deaminase converts non-toxic 5-fluoro-cytosine (5-FC) into 5-fluorouracil (5-FU), which is highly toxic to plants (Perera et al., 1993). Importantly, if added to the germination media, 5-FC allows selection on

plates. We tested the toxicity of 5-FC in our growth conditions, and found that at the concentration of 1mg/ml it allowed tight selection against plants expressing *CodA*, while no toxic effects were observed on plants that did not contain the transgene (Figure 3-14).

Figure 3-14. Seedlings of the PM-55 line on MS medium containing 1mg/ml 5-FC. White arrowheads point at non-sensitive seedlings, black arrowheads point at sensitive seedlings.

Using *Agrobacterium*-mediated transformation, we introduced the construct containing the *CodA* gene and a functional copy of the ORC2 gene (pPM5 construct) into heterozygous *orc2-1* plants. Genetic analysis of 20 transformant families revealed a line that contained a single insertion of pPM5, and that was homozygous for the *orc2-1* mutation (PM55-11).

Although the designed system appears to be stringent and fully applicable for the proposed screen, acquiring it took a long time, and due to the time limitations, the actual screening could not have been performed within the timeframe of my PhD thesis. However, given that the system is now established, it can build a solid ground for a thesis work for another student.

3.4 Candidate gene approach.

In order to identify ORC2 partners, we also used the candidate gene approach. First, based on the similarity of their phenotype, we chose the *TITAN* class mutants (Liu and Meinke, 1998; Liu Cm et al., 2002; Tzafrir et al., 2002) as candidates. The second subset of candidate genes was selected based on the similarity of function with the MEDEA protein. MEDEA is a *Polycomb* group protein that has a function in chromatin modification (Grossniklaus et al., 1998; Guitton and Berger, 2005),

and was shown to genetically interact with ORC2 (Collinge et al., 2004). Based on that, I performed crosses with other mutants in genes encoding proteins associated with chromatin modification. These included *fis2* (Chaudhury et al., 1997), *fis3 (fie) (Ohad et al., 1996)*, *eza-1* (*swn-3*) and the homologue of animal Heterochromatin Protein 1 (HP1), *tfl2/lhp1* (Gaudin et al., 2001). MEA, FIS3 and MSI1 were shown to interact (Kohler et al., 2003), forming the so-called FIS complex that has a function in seed development. The FIS complex, which probably also contains FIS2 protein (Chanvivattana et al., 2004), modifies the expression of its target genes by applying repressive methylation on histone H3 lysine 27.

The genetic interaction between *ORC2* and *MEDEA* was detected as a shift in proportion of seeds that aborted in two different sizes. The amount of small aborting seeds, typically observed in the *orc2-1* mutant, was found to be lower than expected in plants bearing both *orc2-1* and *medea* mutations. I inspected siliques of plants carrying either *ttn2, ttn4, ttn8, fis2, fis3, eza-1* or *tfl-2* mutations together with the *orc2-1* mutation, to look for altered seed abortion phenotypes. I also cleared and inspected aborting seeds microscopically. These analyses, however, revealed neither a shift in seed abortion ratio, nor changes in the phenotype of aborting seeds. The fact that I did not observe the *orc2* mutant phenotype to be modified by *fis2*, *fis3* and *msi1* mutations implies that the effect of *mea* is independent of FIS complex. Such a FIS-independent effect was also observed for auto-repression of the maternal *MEA* allele (Baroux et al., 2006).

3.5 Inducible RNAi system.

If a mutation causes embryo lethality, it means that the affected gene has an essential function in embryo development. However, it says little about the gene function in adult tissues. If we could shut down the expression of the *ORC2* gene later in development, it would be possible to learn about its role beyond embryogenesis. To achieve this, I designed an inducible RNAi system that would allow expression of an RNA hairpin, which should trigger the RNAi response against a gene of interest, here *ORC2*, knocking down its expression.

The pHellsgate8 vector allows for easy introduction of an inverted repeat of a sequence of interest. When the inverted repeat is transcribed, it forms an RNA

hairpin that is able to induce the RNAi response and degradation of the transcripts of the endogenous gene (Smith et al., 2000; Helliwell and Waterhouse, 2005). However, the expression of the repeat in the pHellsgate8 vector is controlled by the CaMV 35S promoter, which is active in most of plant tissues throughout plant development. To be able to express the hairpin only in specific tissues and specific developmental stages, we took advantage of the estradiol-inducible system (Zuo et al., 2000), and exchanged the CaMV promoter for the minimal 35S promoter with a lexA binding site. This promoter becomes active in the presence of XVE, a chimeric transcription factor that enters the nucleus upon addition of estradiol. That is, before estradiol is applied to plants, the hairpin will not be expressed. The Gateway compatible vector was named pPM4i.

I introduced a fragment of the *AtORC2* coding sequence into pPM4i, to produce plasmid pPM4iO2. This construct was introduced into *Arabidopsis* plants, that had previously been transformed with an inducer construct (pMDC17 (Curtis and Grossniklaus, 2003)) that drives the expression of XVE constitutively from the G10-90 promoter (Ishige et al., 1999). In order to monitor the induction level, the inducer construct also contained the *GUS* reporter gene under the control of the min35S-lexA promoter (i.e. the same as the promoter driving the expression of the hairpin). Thus, upon the application of estradiol, not only expression of the hairpin would be induced, but also the expression of the GUS protein, which can be easily monitored in situ.

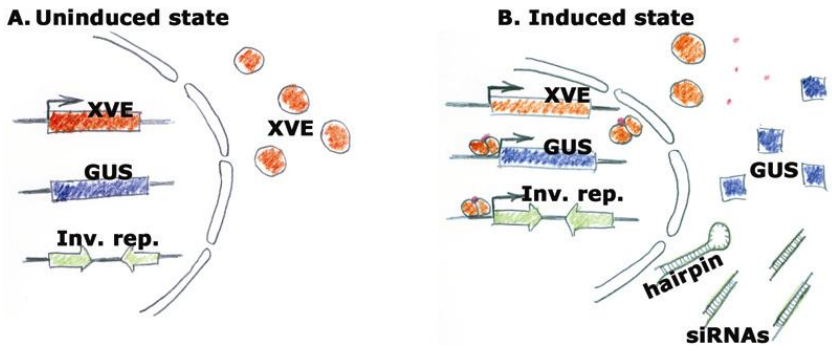


Figure 3-15. The Inducible RNAi system. A. In an uninduced state the XVE transcription factor (orange balls) is present as monomers and does not localize to nucleus. Upon binding of estradiol (**B**), XVE dimerizes and translocates to the nucleus where it binds and activates the expression from inducible promoters.

I isolated three plant lines that exhibited a strong estradiol response (based on results of GUS assay after application of estradiol), and contained a single insertion of the anti-ORC2 RNAi construct. I applied estradiol to plants of these lines, and monitored the resulting phenotype, GUS expression, and the level of the *ORC2* mRNA at different time points after induction. GUS expression was noticeable as early as 12 hours after the induction, but I failed to observe a phenotype distinguishable from the control. After one week of growth on plates containing estradiol, *Arabidopsis* seedlings were loosing shoot and root meristem activity, but a similar phenotype was also observed in control seedlings that grew on plates that did not contain estradiol, but only 0.01% of ethanol, the substance used to dissolve estradiol.

I also tested the level of *ORC2* mRNA in induced and non-induced seedlings by semi-quantitative RT-PCR. I performed these tests with three different sets of oligonucleotides to amplify the signal from the ORC2 mRNA (relative positions of olygonucleotydes used are shown in the Figure 3-16, results are shown in the Figure 3-17). Using sets #2 and #3, I saw no change in the amount of *ORC2* mRNA. However, when the set #1 was used, instead of breakdown, an increased ORC2 signal was observed. This increase can be explained: the fragment of the *ORC2* gene used to create the inverted repeat in the responder construct, was amplified by the oligonucleotide set #1. Thus, upon estradiol induction, the hairpin is transcribed, and the level of RNA containing the sequence that can be amplified by the oligo set #1 is increased. Theoretically, because of the double-stranded nature of the RNA hairpin, it should be degraded immediately after its synthesis by the RNA interference machinery, triggering the RNAi response. However, our results showed that this did not happen. Possible explanations for this will be presented in the discussion chapter of this manuscript.

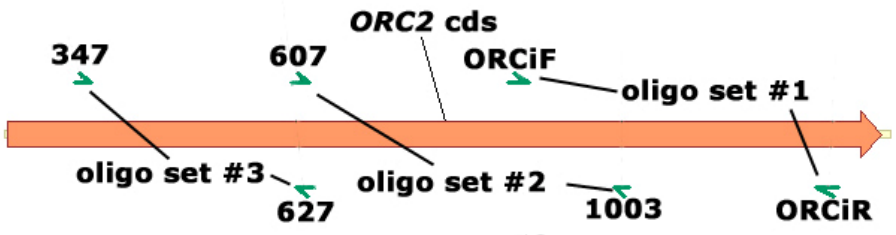


Figure 3-16. Positions of oligonucleotide sets used in relation to the AtORC2 coding sequence. The set #1 was used to generate the inverted repeat in the vector pPM4iO2.

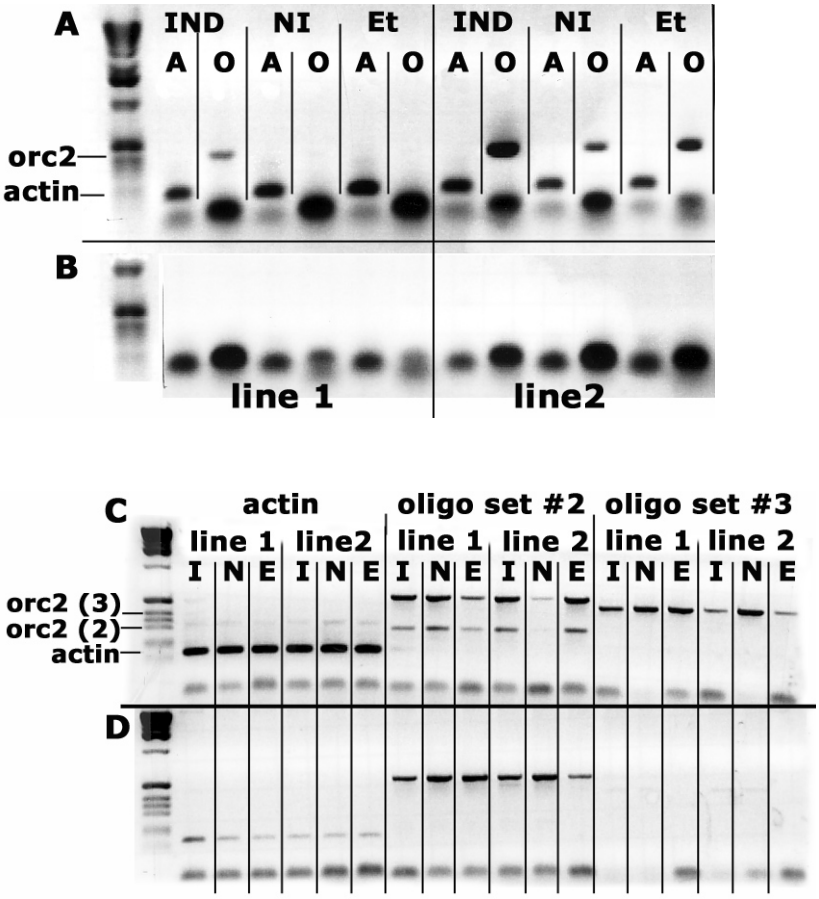


Figure 3-17. Analysis of *ORC2* expression in lines used for inducible RNAi. A. RT-PCR using oligo-set #1 (**O**) (see fig 4-14) and actin 11 primers (**A**) in two different lines. IND – induced plants, NI – non-induced plants. Et – non-induced, but grown on media with 0.01% Ethanol. **B.** Negative control (no reverse transcriptase) for **A**. **C.** As in panel **A.,** but with AtORC2 olygo sets 2 and 3. **D.** Negative control for **C.** Note unspecific bands for oligo-set#2.

4. DISCUSSION.

My results bring clues to understanding the phenotype we observe in the *orc2-1* mutant. Since the homologues of the AtORC2 protein in other organisms were shown to participate in the replication of nuclear DNA and are conserved throughout eukaryotes, it is proposed that the retarded embryo development in the *orc2-1* mutant is largely caused by extreme elongation of S-phase of the cell cycle as the number of licensed origins decreases. Models for how this can lead to disruption of embryo patterning and endoreplication in endosperm nuclei are presented in Sections 4.2 and 4.3. The fact that *orc2* mutant cells divide at all can perhaps be explained by the carryover of ORC2 protein/mRNA that has been synthesized premeiotically or to the production of partially active protein, as discussed below.

4.1 Detailed phenotypic analysis of the orc2 *mutant and differences between mutant alleles.*

The three mutant *orc2* alleles analyzed in this study exhibit similar features: ¼ of seeds abort in plants heterozygous for the insertion, embryos stop their development before reaching the globular stage, and seeds contain enlarged endosperm nuclei. However, clear differences exist between the mutant phenotypes of these alleles, as summarized in Figure 3-4.

4.1.1 Differences in the endosperm phenotype

The least degree of endosperm proliferation was observed in the orc2-2 line (usually 1 to 2 divisions), while in the *orc2-1* and *orc2-3* lines the endosperm went through more divisions. In the *orc2-3* line, the number of endosperm nuclei in some seeds reached values close to that of wild-type endosperm. This was unexpected, as the GT-7766 insertion is located at the beginning of the *ORC2* coding sequence (Figure 3-4) and I expected *orc2-3* to be a null allele. The higher number of

endosperm nuclear divisions cf. *orc2-2* suggests that some ORC2 protein is produced post-zygotically in homozygous mutant seeds of the *orc2-1* and *orc2-3* lines. Both these mutations were caused by the insertion of a *Ds* element, and this presents a possible explanation why the endosperm proliferates more in *orc2-1* and *orc2-3* lines in comparison to *orc2-2*, which is a T-DNA-insertion line.

It is known that *Ds* elements carry cryptic splice sites at their termini, and that, at least in some instances, *Ds* elements can be spliced out of the transcript (Wessler et al., 1987). Although *Ds* element splicing has not yet been reported for *Arabidopsis*, the possibility that it can occur, at least under certain conditions, cannot be excluded. Thus this appears to be a likely reason for the increased endosperm proliferation cf. *orc2-2* in the *Ds*-insertion lines, *orc2-1* and *orc2-3*. Further, differences in splicing efficiency between the two alleles probably also accounts for the difference in the number of endosperm nuclei formed in *orc2-1* cf. *orc2-3*. In contrast, T-DNA is apparently unable to be spliced out of the transcript, since it does not have cryptic splice sites. Thus, it is likely that the endosperm phenotype in the *orc2-2* line represents the mode of endosperm development in complete absence of zygotically expressed ORC2 protein. However, it cannot be excluded that the difference in endosperm development between *orc2-2* and *orc2-1/orc2-3* lines can be also explained by differences in the background phenotype (Landsberg *erecta* for *orc2-1 and orc2-3* and Columbia for *orc2-2*).

4.1.2 Differences in the embryo phenotype

Interestingly, the severity of the embryo phenotype in the three mutant lines did not correlate with the severity of the endosperm phenotype. In *orc2-1* and *orc2-2* lines embryos produced a similar number of cells before seeds aborted (Figure 3-4), and in the *orc2-3* line embryos rarely progressed further than the 2-cell stage. These phenotypes are in accordance with the hypothesis that in the *orc2-1* and *orc2-2* lines, in which insertions are located at the end of the coding sequence, some truncated ORC2 protein is produced. Such truncated semi-functional proteins might allow embryos in these lines to proceed further than in the *orc2-3* line, where the insertion disrupting the gene is located at the beginning of the coding sequence.

The phenotypic differences between embryo and endosperm indicate that the putative splicing of the *Ds* element from the ORC2 transcript must occur in a tissue-specific manner: and that splicing events, particularly of the *orc2-3* allele, occur in the endosperm, but not in the embryo. Indeed, *Ds* element excision in maize has been shown to be tissue-specific: the splicing pattern in maize endosperm was different to splicing of the same element in suspension cell culture (Lal and Hannah, 1999). The reported tissue-specificity of *Ds* splicing could thus be an explanation for the observed difference between embryo and endosperm in the severity of the *orc2-3* phenotype.

In some cases the spliced out *Ds* element leaves a footprint in the mRNA, making mutant transcripts distinguishable from the wild-type transcripts of a gene (Lal and Hannah, 1999). A functional protein can still be translated from such a mutant mRNA if the footprint did not create a premature stop codon or frame-shift. I was not able to amplify any different species of *ORC2* mRNA in seeds. The failure to detect such species, however, does not exclude this possibility. First, it is possible that the spliced out element does not leave any footprint at all, or the footprint size is only 3 nucleotides, which would be undetectable in our experiments with maximal resolution of 5-10 nucleotides. Secondly, the abundance of such an mRNA is likely to be very low, and its presence could be masked by the vast excess of wild-type *ORC2* mRNA originating from maternal tissues. Laser-assisted microdissection of mutant endosperm would be necessary to test for and/or prove the presence of functional *ORC2* mRNA in the mutant endosperm of the *orc2-3* line.

4.2 Model for the observed orc2 *mutant embryo phenotype.*

My results show that embryo development in all the three alleles of *orc2* is substantially slowed down, and cell divisions occur in non-characteristic planes for *Arabidopsis* development. As has been noted in the Introduction, this phenotype is not unique and a number of *Arabidopsis* mutants exhibit similar developmental abnormalities. The appearance of abnormal division planes in early embryo development has been described in a number of mutants. In mutants, such as *gurke* (Torres-Ruiz et al., 1996), *fass* (Torres-Ruiz and Jurgens, 1994), and *gnom*

(Meinke, 1985), division planes are abnormally positioned starting already from the first division of the zygote. Genes affected by these mutations code for acetyl-CoA carboxylase (*gurke*) (Baud et al., 2004), a PP2A subunit *(tonneau2/fass)* (Camilleri et al., 2002) that is involved in microtubule cytoskeleton organization (McClinton and Sung, 1997), and a regulator of intracellular trafficking (*gnom*) (Shevell et al., 1994) involved in auxin transport (Steinmann et al., 1999). The role of these genes in cell polarity determination is now well understood. Notably, mutants for these genes are able to develop embryonic organs, albeit abnormal ones, and germinate into aberrant, but adult plants. This means, that although these genes are important for plant development, they are not ultimately essential for plant survival.

Another set of mutations exists in which the division planes in embryogenesis are improperly positioned, but our current knowledge does not allow the establishment of a direct link between their function and cell polarity determination. These mutations include *prolifera* (MCM7 replicative DNA helicase) (Holding and Springer, 2002), an alanyl-tRNA synthetase gene (Ge et al., 1998), members of the ubiquitin/26S proteasome pathway (Doelling et al., 2001; Brukhin et al., 2005), a cellulose synthase-like glycosyltransferase (Goubet et al., 2003), replicative DNA polymerase epsilon (Jenik et al., 2005), DNA polymerase theta (Inagaki et al., 2006), and *orc2* (Collinge et al., 2004). It is surprising that disruption of such a variety of cellular processes leads to very similar phenotypes. Certainly, it cannot be excluded that the aforementioned genes have a direct function in cell polarity determination. However, it appears likely that their dysfunction has a common feature that leads to the appearance of this phenotype. The molecular nature of this feature is elusive, but in all of the aforementioned mutants embryos develop slower than in wild-type, and the inappropriate timing itself may be the reason for mis-positioning of division planes, as discussed below.

Indeed, the timing of divisions in embryo development appears to be an important factor for at least a number of living organisms. In plants these processes have been very scarcely studied so far, but in mammals, the mechanisms of spatio-temporal regulation of embryo development appear to be well understood. For example, the formation of somites in vertebrates can happen only within a short time frame and is controlled by an oscillator, the so called segmentation clock, driven by Notch and Wnt cascades (reviewed in (Andrade et al., 2005).

Some reports suggest that a correlation between the developmental process and its timing also exists in the plant kingdom. In maize leaf patterning, timing is believed to be a factor in specification of developmental fates by different cell lineages (Muehlbauer et al., 1997; Osmont et al., 2006). In *Arabidopsis* embryogenesis, the correlation between the timing of division and the division plane positioning, is likely to exist too. The formation of the root primordium is affected in a hypomorphic mutant of a subunit of *Arabidopsis* DNA polymerase ε (Jenik et al., 2005). The cell cycle in developing embryos is slowed down by about 35% and the hypophysis frequently divides in the wrong orientation or asymmetrically leading to an aberrant root pole. Also, *Arabidopsis* plants presumably having a prolonged G2 phase because of lack of the TEBICHI protein, a homologue of the mammalian DNA polymerase theta, exhibit abnormal division planes in both embryogenesis and meristems (Inagaki et al., 2006). In the *orc2* mutant, embryos develop much slower than their siblings, and, before they arrest, do not follow the strictly defined pattern of early *Arabidopsis* embryo divisions. Does ORC2 have a direct function in cell plate positioning? Or is another, common but yet unknown mechanism affected in this mutant, the disruption of which makes embryos in a vast majority of cell-cycle mutants undergo non-characteristic cell divisions? The number of mutations producing similar phenotypes, as well as the fact that cell division planes in them are positioned randomly at a variety of stages of embryo development makes the latter possibility more realistic.

The common feature among these mutations is that embryos develop slower than in wild-type. This could mean that some general mechanism exists in *Arabidopsis* (here termed “positional clock”) that positions division planes depending on the timing of embryo development. If there were such a positional clock, then the reason for the occurrence of abnormal division planes in the *orc2* mutant embryos, as well as in embryos defective for other genes involved in the progression of the cell cycle, would not be that these gene products are directly involved in the positioning of the division plane, but rather that the cell reaches mitosis at the wrong time, i.e. when the positional clock has moved on and sets the division plane in a position appropriate for a later stage of embryo development. A few mutations have been described in which embryo development is slowed down, but the patterning is not affected (Xu et al., 2005; Gomez et al., 2006). A common

feature of these mutations is that they affect general metabolic pathways. Possibly, in these mutants, the positional clock is slowed down together with the cell cycle.

It is difficult to speculate currently what might be the nature of the positional clock proposed here. WOX genes were shown to be expressed differentially in different domains of a developing embryo (Haecker et al., 2004). However, expression of these genes is more likely to be the effector, driving the specification of cell fates. A more likely candidate for the clock is the loop of auxin flux that drives the expression of the PIN auxin transporters, whose specific intracellular localization at a particular cell wall effects the polar transport of auxin (Vieten et al., 2005; Sauer et al., 2006). Polar auxin transport can control cell division planes (Dhonukshe et al., 2006), and is important for early embryogenesis (Tanaka et al., 2006). The slowing of the cell cycle and asynchrony between adjacent cells may perturb the regulated auxin flux within the embryo and cause the irregular divisions we and others observed.

In plants, the position of the division plane is determined before the mitosis by the preprophase band (PPB) of microtubules that disappears by prometaphase, leaving a modified region of the cell membrane, the so-called KCA-depleted zone (KDZ) (Vanstraelen et al., 2006). The KDZ position can be monitored, and if the positional clock indeed exists in *Arabidopsis* embryogenesis, then in mutants with slowed down progression of the cell cycle a migration of KDZ should be observed. Such experiment, thus, can be performed as a test for the existence of the clock proposed here.

4.3 Endosperm endoreplication and chromatin structure.

In early endosperm development in wild-type *Arabidopsis*, nuclear divisions occur synchronously (Yakovlev, 1976). This is not surprising given that the endosperm in an early seed is a single multinucleate cell, in which the cell cycle should progress at a similar pace around all the nuclei. However, if the S-phase of the cell cycle progresses at a different pace in different *orc2* mutant endosperm nuclei, the signal for starting mitosis would be given to nuclei that had not completed S phase. This would lead to an attempt to separate incompletely

replicated chromatids, which would be prevented by bridges of non-replicated DNA connecting sister chromatids.

Secondly, hampered DNA replication might have a direct effect on chromosome compaction, which is essential for successful mitotic chromosome segregation. A model directly connecting replication and chromosome condensation has been proposed (Pflumm, 2002). In this model the replication forks from one origin are connected to each other, so that replicated strands of DNA emerge from the complex of two replicative forks as two loops. The model also proposes that chromatid cohesion and compaction are established co-replicatively. The length of replicons would thus influence the chromosome compaction, i.e. the longer (and more rare) replicons are, the bigger will be the loops emerging from the DNA replication site, and hence, the thicker and longer the condensed chromosome will appear. The phenotype observed in ORC mutants in *Drosophila* supports this theory (Pflumm MF, 2001).

Both of these scenarios (incompletely replicated or insufficiently condensed DNA) would lead to the formation of a partially or truly polyploid nucleus that would be driven into the next S-phase to further increase its DNA content. Of course, this hypothesis also requires that the S/G2 transition and/or mitotic checkpoints must be absent in the syncytial endosperm. The latter has not been demonstrated so far, but the existence of a wide range of *Arabidopsis* mutants with giant endosperm nuclei (Liu and Meinke, 1998; Mayer et al., 1999; Holding and Springer, 2002) supports this possibility.

The formation of giant endosperm nuclei seen in mutants with cell division defects, including *orc2*, would thus occur when DNA replication has not been accomplished at the time when the signal for mitosis appears; and when chromosomes cannot be separated during mitosis. The latter can be caused either by a failure to condense chromosomes properly (as in the case of condensin and cohesin mutants (Liu Cm et al., 2002), or because of the lack of the cytoskeleton machinery necessary for chromosome segregation (McElver et al., 2000; Steinborn et al., 2002; Tzafrir et al., 2002).

The heterogeneity in sizes of enlarged endosperm nuclei, commonly observed in mutants exhibiting this phenotype, can be explained by the asymmetrical distribution of the maternally-loaded, functional protein between dividing nuclei.

Differential distribution of a replication protein between the daughter nuclei would allow nuclei that acquired more protein to accomplish S-phase earlier. Such nuclei will start releasing mitotic cyclins into the common cytoplasm of the endosperm tissue, and force other nuclei into an M-phase that cannot be accomplished because the DNA has not been completely replicated.

4.4 AtORC2 and chromatin structure

In yeasts and metazoa, ORC2 protein was also shown to have a role in heterochromatin formation and in chromosome condensation (Triolo and Sternglanz, 1996; Pflumm MF, 2001; Prasanth et al., 2004). The data suggests that this function is also preserved in *Arabidopsis*. FISH on the giant endosperm nuclei of the *orc2-1* mutant reveals that normally heterochromatic regions of chromosomes, the centromeres and nucleolar organizers, were largely decondensed and appear dispersed in mutant nuclei. However, since we never observed mutant endosperm nuclei undergoing mitosis, it is not possible to say whether mitotic chromosome condensation is disrupted along with constitutive heterochromatin structure, although it is a likely possibility. Lack of proper condensation (as well as incomplete replication of nuclear DNA) in *orc2* is likely to lead to the formation of giant endosperm nuclei.

4.5 Zygotic lethality of the orc2 *mutant*

The inability of homozygous *orc2* plants to accomplish embryogenesis clearly shows that the *AtORC2* gene is essential for *Arabidopsis* development. Considering its essential function in the cell cycle, it is very likely that it is also essential for gametophyte development, which includes 2 and 3 rounds of mitotic divisions to produce the mature male and female gametophytes respectively. However, we did not observe any evidence for gametophytic defects in of the *orc2* mutants.

This paradox is a common situation in *Arabidopsis*: mutations affecting components of the cell cycle are rarely gametophytically lethal. For example, mutations in essential genes such as MCM7 (prolifera) (Springer et al., 1995), AESP (*Arabidopsis* homologue of separase) (Liu and Makaroff, 2006), SMC1 and SMC3 cohesins (*ttn7* and *ttn8*) (Liu Cm et al., 2002) result in zygotic lethality. However, in *Arabidopsis* very few mutations were described that affect a gene necessary for the progression of cell cycle and lead to gametophytic lethality. One example is the mutation in the *AGM* (*ABNORMAL GAMETOPHYTES*) gene that encodes a transmembrane protein with a function in mitosis (Sorensen et al., 2004). A number of explanations exist for this apparent paradox.

The first explanation is that such mutations can be difficult to recover. Indeed, if the mutation affects both male and female gametophytes, then the mutant allele will not be transmitted to the progeny. Secondly, gametophytes are substantially different from other plant tissues and it is possible that many genes that are essential for sporophyte development are not necessary for gametophytes. Microarray data shows that only one third (29%) of *Arabidopsis* genes are expressed in mature pollen (Pina et al., 2005). However, this explanation does not seem to apply to the ORC2 gene, because, according to expression profiling experiments (Honys and Twell, 2004), as well as to my data showing the expression of the AtORC2:GFP fusion construct (section 3.2.3), *AtORC2* is expressed in both male and female gametophytes.

The third possible explanation to the paradox comes from the notion that many plant genomes, including *Arabidopsis*, are duplicated (McGrath et al., 1993; Kowalski et al., 1994), and hence, a certain degree of redundancy for a given gene exists in the haploid generation. This obviously does not apply for *AtORC2*, which is a single gene in *Arabidopsis* (Masuda et al., 2004). And finally, although not yet directly shown for *Arabidopsis*, it is very likely that many mRNAs/proteins that are found in gametophytes, are synthesized premeiotically or transferred to the developing gametophyte from surrounding maternal tissues. I attempted to determine whether high levels of the ORC2:GFP construct are present in the Megaspore Mother Cell, but it was not possible, because endofluorescence in the green range is especially strong in the female reproductive organs at this stage (my results, and C. Baroux, personal communication), prohibiting studies of GFP fusion proteins.

4.6 Localization of the AtORC2 protein and its role in plant reproductive development.

To learn more about the function(s) of AtORC2 in chromosome integrity and inheritance, I determined its localization during the life cycle and within the cell. The data I obtained using the ORC2:GFP construct, confirms and expands the data obtained in (Collinge et al., 2004) on GUS expression from the enchancer detector that inserted into the ORC2 gene, and by *in situ* RNA hybridization on ovules. The functional AtORC2:GFP construct showed that AtORC2 is expressed not only in pollen, trichomes and ovules, but also in dividing tissues, such as leaf primordia and the root apical meristem. The expression in the meristem was not observed in the enhancer detector line, but it is consistent with the function of ORC2 as a replication protein regulated by E2F (Diaz-Trivino et al., 2005).

4.6.1 Karyogamy and ORC2 localization in the male gametophyte

At karyogamy, gametes have to be at the same stage of the cell cycle in order to ensure that genomes of both parents contribute equally to the offspring. As reviewed in chapter 1.5, sperm nuclei in *Arabidopsis* were reported to reach G2 phase at fertilization, and the egg and central cell are presumed to be at the same cell cycle stage (Friedman, 1999). In this respect, the localization of the AtORC2:GFP protein in pollen was surprising. In *Arabidopsis*, the mature pollen grain consists of three cells, a vegetative cell and two sperm cells. Our observation of mature pollen grains and growing pollen tubes revealed that ORC2 localizes to the vegetative nucleus, but not to the nuclei of the sperm cells, where we would expect to see it according to the published data that sperm nuclei are in S phase from the period beginning immediately after their inception until their arrival in the degenerating synergid (Friedman, 1999). Such localization is surprising, considering the data presented in Chapter 3.2.2, which suggests that ORC2 is nuclear localized through the cell cycle, and leaves DNA only in mitosis.

A number of explanations can be proposed to explain this contradiction. First, the experimental systems were different: I performed pollen germination experiments in vitro, while Friedman made observations on pollen tubes growing inside a flower. However, Friedman's data shows that sperm nuclei enter S-phase shortly after the generative cell mitosis, and in the majority of tricellular pollen grains sperm cells have entered S-phase. The ORC2 distribution pattern we observed in tricellular pollen was mostly identical to the pattern observed in pollen tubes, and thus, our inability to detect ORC2 in sperm nuclei is not likely to reflect differences in the experimental system. The second possibility is that AtORC2 may not be required for sperm cell replication. Thirdly, ORC dynamics may be different in plant gametophytes compared to somatic cells: such differences have been observed in studies of *Xenopus* ORC in egg extracts. In this experimental system, after the assembly of the PreRC, the affinity of *Xenopus* ORC for sperm chromatin is merely reduced, but after PreRC assembly on somatic cell chromatin, ORC leaves the chromatin completely (DePamphilis, 2005). If ORC dynamics are different in *Arabidopsis* between sperm and sporophyte chromatin, then after licensing the origins in generative cell M-phase, ORC2 might immediately leave the sperm chromatin, and become degraded. Thus, it is possible that the interaction between ORC and the sperm chromatin to license origins of replication occurs within a very short time frame that eluded our observations. To detect this time point between generative cell mitosis and entry of sperm nuclei into S-phase, further studies of pollen development are necessary.

4.6.2 ORC2 in female gametophyte

The highest levels of AtORC2:GFP were observed in the female gametophyte, and in seeds shortly after fertilization. This is not surprising, considering that early embryogenesis in many organisms, including *Arabidopsis*, is characterized by a dramatically accelerated cell cycle (Glover, 1989; Mac Auley et al., 1993; Boisnard-Lorig et al., 2001). Such acceleration requires large amounts of replication proteins. Faster progression of the cell cycle, and S-phase in particular, requires that the genomic DNA has to be replicated faster. However, since the speed of the DNA polymerase complex is relatively constant, the only way to speed this process

up is to increase the density of replication forks, thus decreasing the size of a replicon (DePamphilis, 2000). This would likely require a larger population of replication proteins to be present in the zygote. It is well known that animal egg cells accumulate proteins and mRNAs in order to ensure that the zygote can proceed through a few rounds of cell divisions before the zygotic transcription starts. It is likely, although not directly shown, that plant gametes also inherit and rely on maternally synthesized proteins/mRNAs. An analysis of GUS expression from the gene trap inserted into the *PROLIFERA* gene revealed that the female gametophyte accumulates PROLIFERA protein or mRNA prior to fertilization (Springer et al., 2000). It is not yet clear whether these products are of sporophytic or gametophytic origin.

Notably, distribution of the ORC2:GFP protein in the female gametophyte was similar to the pattern observed for the *PROLIFERA* gene product. In immature ovules, as well as in the central cell and the egg cell we observed increased expression levels of the ORC2-GFP fusion protein. Accumulation of ORC2 in the egg and in the central cell reflects the significant either premeiotic or postmeiotic maternal contribution to the early embryo and endosperm, allowing fast progression of first divisions of these tissues after fertilization. In accordance with the fact that in the female gametophyte only the egg and the central will grow and contribute to the seed, very little ORC2 was detected in the synergids and antipodals of mature embryo sacs, in contrast to the situation within immature ovules, where all eight nuclei had equal level of ORC2:GFP protein.

The observed difference in the amounts of maternally and paternally provided ORC2 protein suggests that some maternal effect could exist in the *orc2* mutant. However, I did not observe any substantial difference in development between developing embryos in a silique of heterozygous plants. This suggests that transcription of the paternal copy of the *ORC2* gene starts very early after fertilization and/or that pollen carries ORC2 mRNA, but also brings an indirect evidence for the presence of ORC2 transcripts/protein in the female gametophyte that is synthesized premeiotically.

4.7 Dynamics of AtORC2 during the cell cycle

During S-phase, the nuclear DNA has to be replicated completely but only once. Eukaryotes solve this problem by inactivating the Pre-RC immediately after it promoted replication. Such inactivation is performed by modifying, inactivating or removing members of the Pre-RC from the nucleus (reviewed in (DePamphilis, 2005)). Our data shows that in *Arabidopsis* ORC2 colocalizes with DNA during interphase, but leaves chromosomes during mitosis. This contrasts to the situation observed in yeasts, *Drosophila* and mammalian cells, where ORC2 is a member of a stable complex associated with the chromatin throughout the cell cycle. Removal of the AtORC2 protein from the DNA may be a part of the mechanism preventing re-replication. It has been proposed that plants utilize removal of the replication machinery from the nucleoplasm to regulate cell cycle progression. MCM6 protein of maize was shown to accumulate in the nucleoplasm during the G1-phase, and leave the nucleus during the S-phase (Dresselhaus et al., 2006). MCM7 protein in *Arabidopsis* remains in the nucleus throughout interphase, but is not associated with chromatin during mitosis (Springer et al., 2000). AtORC2, according to my data, behaves in a similar manner, bringing further evidence that physical removal of PreRC components from the chromatin is common to *Arabidopsis,* and is possibly a part of the mechanism preventing re-replication. Another feature that highlights differences in ORC regulation between Arabidopsis and animals is that expression of most *Arabidopsis* ORC subunits (with the exception of ORC5) is regulated by the E2F transcription factor (Diaz-Trivino et al., 2005), while in animals only ORC1 is subject to E2F control (Ohtani et al., 1996; Asano and Wharton, 1999). Together, these results suggest that the control of initiation of DNA replication in plants differs substantially from that of the animal kingdom.

4.8 Partners of the ORC2 protein

A recurring theme arising from the study of ORC proteins is that, although they are conserved throughout eukaryotes, their regulation and even function have diverged to fit the requirements of different phyla and species. For example, the

persistence of the ORC on chromatin through the cell cycle, the mode of regulation and even the composition of the complex differ significantly (DePamphilis, 2003; Kearsey and Cotterill, 2003). Regarding function: there are additional roles of different subunits and subcomplexes outside origin licensing (Prasanth et al., 2002; Chesnokov et al., 2003; Prasanth et al., 2004; Huang et al., 2005). Even in replication licensing, while yeast ORC recognises origins of DNA replication by sequence, in multicellular eukaryotes other features govern origin identity, and other proteins may be required for recruiting ORC to origins (Cvetic and Walter, 2005). Thus, it appears likely that in *Arabidopsis* the PreRC can include previously unidentified proteins.

In the course of my thesis work I attempted to identify partners of the ORC2 protein in *Arabidopsis*. Two strategies were employed. First, I performed crosses between plants heterozygous for the *orc2* mutation and other selected mutants. The selection was based on either similarity of the mutant phenotype (e.g. giant endosperm nuclei), or similarity of the known function (e.g. chromatin protein). Secondly, as stated in chapter 1.10, I intended to perform a screen for a suppressor of the *orc2* phenotype.

Both strategies are similar in the sense that the feature I was looking for was a suppression of the *orc2* mutant phenotype. Suppression might ocuur in the following cases:

1. The mutation might positively regulate ORC2 expression (e.g. by changed splicing activity of alleles in which a transposon has inserted into AtORC2) thus increasing the level of ORC2 protein in the *orc2* mutant background.

2. The mutation causes an increased level of a protein normally activated by ORC2.

3. The mutation occurs in a protein normally activated by ORC2, so that the modified protein acquires its active state in the absence of ORC2 protein.

4. The mutation occurs in a non-ORC pathway, which allows ORC to remain functional in the absence of the second subunit of the complex (ORC2). For example, a mutation leading to a change in

chromatin structure, allowing ORC1+ORC3-6 complex to bind origins, that are inaccessible otherwise.

5. The mutation occurs in a non-ORC pathway that allows the cell to initiate genome replication completely bypassing the ORC pathway.

If both ORC2 and its partner (protein X) are necessary for a given process, then mutation of the protein X in the *orc2* mutant background is unlikely to rescue the *orc2* mutant phenotype, since the pathway in this case would remain blocked. Thus, my failure to detect a partner of ORC2 using the candidate gene approach does not mean that ORC2 does not interact with the proteins I analyzed (FIS2, FIS3, LHP1, EZA1, TTN2, TTN4, MSI1), but only means that mutations in these particular genes in *orc2-1* background do not restore the pathway ORC2 is involved in.

A large-scale screen for suppressors of the *orc2-1* phenotype seems to be a necessity to identify partners of the ORC2 protein *Arabidopsis*. In the course of my study I established the system for performing such a screen. The screening strategy is based on the selection of seedlings that are able to grow in the absence of ORC2 protein. This selection would be achieved by negatively selection for a marker, *CodA,* linked to a functional ORC2 transgene. The negative selection (with 5-FC) has been proven to be tight. Thus, I estimate that among mutagenized seedlings only those that contain a mutation either in the *CodA* gene (negatively selectable marker), or in genes encoding for partners of ORC2 will be able to survive in the presence of 5-FC.

However, although screening for a plant containing a suppressor mutation appears a fast and easy task, mapping the mutation responsible for the suppression of the *orc2* mutant phenotype would have taken time unavailable for my PhD study. However, the system I established builds a solid ground for another PhD project, and I hope that another student would take over this project and succeed in finding unique partners of ORC2 in *Arabidopsis thaliana*.

4.9 Inducible knockdown

RNA interference (RNAi) is defined as the process in which mRNA can be specifically degraded by the cellular machinery upon the introduction of double-stranded RNA (dsRNA) of the corresponding sequence (Fire et al., 1998; Baulcombe, 2005). In recent years RNAi proved itself to be a powerful tool in knocking down expression of genes of interest in a variety of living organisms. The ability to knock down expression of a gene of interest without a necessity to obtain a mutant is a valuable tool in reverse genetic studies. In studies involving *Arabidopsis* and rice, the use of RNAi-mediated knockdowns might be considered limited, given that insertional mutants are easily obtainable from stock centers for many genes, but this technique offers a number of unique features, making it advantageous:

- The expression of a gene is not abolished, but rather the amount of mRNA is dramatically reduced, allowing generation of mild, non-lethal phenotypes.
- Several genes sharing sequence similarity can be knocked down at once (Miki et al., 2005; Raynaud et al., 2005). This feature is especially valuable in plant research, since many plant genomes, including *Arabidopsis*, underwent duplications and contain functionally redundant genes.

The disadvantages include:

- Variability of the phenotype produced depending on the transgene position, its sequence, etc.
- The transgene encoding the hairpin can get silenced after a number of generations.
- The dominant nature of RNAi-mediated knockdowns might preclude the recovery of transformants for the hairpin if it is targeted against an essential gene and the expression of the hairpin causes a lethal phenotype.

While the first disadvantage is hard to fight and is usually solved by analysis of a number of lines carrying the RNAi-inducing transgene, the latter two can be eliminated with the use of an inducible RNAi system. Indeed, if the RNAi construct is not transcribed unless it is induced, it should be less prone to silencing, and also plants carrying such a transgene will be phenotypicaly normal until induction. A further advantage is that induction can allow study of function of essential and/or pleiotropic genes at specific time points or in specific tissues.

In plants, the RNAi response is commonly achieved by introducing a transgene that produces hairpin RNA containing a region of dsRNA. A fragment of a gene of interest is introduced into a vector as an inverted repeat with a spacer in between repeats, which is essential for the stability of the inverted repeat in *Escherichia coli* cells (Wesley et al., 2001). The emergence of inducible systems allowed the more or less precise control of the expression of the RNA hairpins, thus making possible an induction of the RNAi response at the desired time point within development (Chen et al., 2003; Guo et al., 2003; Moore et al., 2006).

I attempted to take advantage of the estradiol-inducible system, well established in our lab (Zuo et al., 2000; Brand et al., 2006), and combine it with pHellsgate8 (Helliwell and Waterhouse, 2003) vector that allows for easy introduction of hairpin-encoding sequences into plant genome. I used a 0.5kb fragment of *ORC2* coding sequence for construction of the inverted repeat. Its expression was under control of the min35S-lexA inducible promoter. This promoter is activated when an active form of XVE, a chimeric transcription factor, is bound to it. XVE expression was driven constitutively by a 35S promoter, but this protein acquires its active state only in the presence of estradiol. Thus the hairpin is expressed only when the plants are exposed to estradiol.

As described in section 3.5, I did not observe a substantial breakdown of the *ORC2* mRNA in my studies. Theoretically, the failure to induce anti-ORC2 response could have occurred for the following reasons:

1. Poor penetration of estradiol into plant tissues
2. Extremely low expression level of the activator protein (XVE)
3. Inappropriate sequence of the responder construct (either in the promoter element or in the sequence of the hairpin)

The first two issues do not seem to be the case, as we observed induction of 35SminLexA::GUS expression upon estradiol application. The *GUS* gene in our experiments was under control of the same promoter element as the anti-ORC construct and was introduced into plants together with the activator construct. A mistake in the sequence of the anti-ORC2 construct is also unlikely, as the plasmid was purified from the *Agrobacterium* clone that was used for plant transformation, and sequencing did not reveal modifications in the pPM4i-O2 vector. Also, since I observed an increase in the abundance of the ORCi fragment (amplified by the *oligo set #1*, Figure 3-16) in induced plants, I believe that the hairpin expression was induced. However, this induction did not lead to breakdown of *AtORC2* mRNA.

A possible explanation for this might be connected to the fact that GUS levels in induced plants were extremely high. If the anti-*ORC2* hairpin was also expressed at similarly high levels, the amount of the hairpin may have saturated the cellular RNAi machinery, so that most of it was used in degrading the hairpin RNA, and degradation of mRNAs was blocked. Indeed expression levels induced by the estradiol/XVE system are extremely high - higher that achieved with the strong 35S promoter (Mark Curtis, personal communication). It may be possible to override this problem by adjustment of the amount of estradiol used to induce the RNAi response. Extremely low doses of estradiol might trigger production of the hairpin in catalytic quantities, leading to degradation of the *ORC2* transcript.

Secondly, it is not entirely clear whether the inverted repeat we used in the pPM4i-O2 vector was folded into a hairpin structure that is able to induce RNAi response. To ultimately prove that the RNAi response is indeed triggered in these lines, it would be necessary to identify whether short RNAs are produced in plants upon induction.

Overall, this inducible RNAi system, if it is well adjusted and triggers a potent response, would undoubtedly be a very useful tool in plant research, that would allow to identify previously unknown functions of genes, mutations in which causes embryo lethal phenotypes.

CONCLUDING REMARKS

Within the course of my PhD thesis, I attempted to fulfill all the goals that were originally set in the beginning of the project (Section 1.10). Unfortunately, such valuable parts as the screen for suppressors of the *orc2* phenotype and the introduction of the inducible RNAi system, could not be completed because of the limited time-frame of my thesis. However, other parts, such as the detailed characterization of the *orc2* mutants and the localization of the ORC2 protein in vivo, have been successfully performed and yielded valuable insights. The results led me to develop the main result, as I see it, of this work: the development of the model for the phenotype observed in *orc2* mutants. Notably, this model can also be applied to a wide range of *Arabidopsis* mutations and thus appears to be a valuable contribution to research of plant development.

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5. APPENDIX

5.1. Oligonucleotide sequences

ORC2gsGAT: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GAA CGG GAG AAC AACT GAT GGG

ORC2gaGAT: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCC CAT TAT CAA ACC GAA TCC C

LexA1d: TGA GCT CCG GTG TCA TCT ATG TTA CTA GAT CGG

LexA1r: TGA GCT CCG GTG TCA TCT ATG TTA CTA GAT CGG

PH1r: TCT TCG TCT TAC ACA TCA CTT GTC

pmPH2d: TGA GCT CCC TAG GTT CAT TTG GAG AGG ACA CGC

ORCiF: TTG TGT CCA TTG GCA GTG TGG GAC A

ORCiR: ATA TTC AAA CAC TCT TGA CCA TCG GA

347F: GAG TAA GCG TTC TGC CCA C

627R: TTA CGG AAG GGA GGT AGC C

607F: ATG GCT ACC TCC CTT CCG

1003R: GTT TGG GCT GTG CTT CCC

DS3.1: CGA TTA CCG TAT TTA TCC CGT TCG

DS5.1: CCG TTT ACC GTT TTG TAT ATC CCG

Orc2gaAsc: TGG CGC GCC ACT GAT TGA GAT CAA GCA AAA GCT G

Orc2gsPac: CGT TAA TTA AAC GGG AGA ACA ACT GAT GGG

5.2. Supplementary chart 1.

stage	*orc2-1*				*orc2-2*				*orc2-3*			
	En	Ep	Su	*	En	Ep	Su	*	En	Ep	Su	*
globular	2	4	4		2	2	2		23	1	2	
	1	2	3		4	4	3		30	1	2	
	0	4	3		3	2	3		35	2	3	
	4	4	3		3	2	3		36	2	2	
	3	2	2		1	2	2		25	2	3	
	4	4	3		1	2	2		12	1	3	
	2	4	3		3	3	4	A	39	1	2	
	0	4	3	W	2	2	3		15	1	2	
	2	4	3	W	3	2	2		21	1	2	
	2	2	4		0	2	1					
	4	2	2	W	1	2	2					
	4	4	3	W	3	2	2					
	4	4	3	W	2	2	2					
	3	4	2	W	1	1	1					
	5	4	4	W	3	1	1					
	3	4	4		5	2	2					
	2	2	2		3	2	2					
	7	6	3	WA	5	2	2					
	2	4	3	W	3	2	2					
	2	4	3		0	2	1	W				
	8	4	3		0	2	2	W				
	4	4	3	W	0	2	2	W				
	2	3	3	A	4	2	2					
	2	3	3	A	1	2	2					
	2	2	2		2	2	2					
	7	4	3		2	1	1					
	3	4	3	W	0	2	2	W				
	3	4	3	W	2	1	2					
	6	4	3	W	0	2	2					
	1	2	2		2	1	1					
	7	4	3		2	1	1					
	4	3	3	A	2	2	2					
					2	2	2					
					2	2	2					
					2	2	2					
					3	2	2					
					4	2	2					
					2	2	1					
					2	2	3					
					2	2	2					
					4	2	2					
					3	2	2	W				
					3	2	2					
					0	2	3					
					3	2	1					
					1	2	2					
					1	2	2					
					3	2	3					
					1	3	3	WA				
					2	2	3					
					2	4	3					
					3	2	2					
					3	2	3					
					2	2	2					
					0	2	3					
					2	4	3					
					1	2	1					
					3	2	2					
					2	2	2					
					3	2	2					
					2	2	3					
					4	2	2					
					5	2	3					
					2	1	2					
					3	2	2					
					0	2	2					
					3	2	2					

Raw data of seed abortion phenotypes in *orc2* alleles. Each row represents a single seed. The numbers of cells in endosperm (**En**), embryo proper (**Ep**) and suspensor (**Su**) are provided for all three alleles. The stage of embryo development of wild-type siblings is indicated in the first column. The column headed with an asterisk (*****) shows if the embryo cells divided in a non-characteristic plane (**W**) and/or asynchrony in embryo proper divisions was observed (**A**).

	orc2-1				*orc2-2*				*orc2-3*			
stage	En	Ep	Su	*	En	Ep	Su	*	En	Ep	Su	*
Globular					3	2	1					
					2	2	2	W				
					1	1	1					
					1	1	1					
					2	2	2					
					2	2	2					
Late Glob.	7	4	8		6	4	3	WA	7	1	2	
	4	6	4	A	1	3	3	WA	10	3	2	A
	4	4	3		2	3	2	WA	20	2	5	
	6	4	3	W	2	4	3		25	2	3	
	2	4	3		2	4	3		23	1	2	
	5	6	3	WA	2	1	2		15	1	2	
	2	5	3	WA	2	2	3		40	4	4	
	9	4	3		1	2	2		4	2	2	
	0	6	5		2	2	2		40	2	2	W
					0	4	3	W	15	2	2	
					3	4	3	W	21	1	1	
					5	4	3		25	1	3	
					1	4	2	W	11	1	2	
					3	4	3		35	2	3	
					2	2	3		30	3	2	A
					3	2	2		12	1	2	
					2	4	3					
					6	4	3					
					1	4	2					
					2	4	3	W				
					1	4	3	W				
					0	4	3	W				
					4	2	2					
					2	1	2					
					3	4	2					
					3	4	2	W				
					1	2	3					
					3	2	3					
					1	2	2					
					0	2	2					
					1	2	3					
					1	4	3	W				
					0	2	2					
					4	2	2					
					1	2	2					
					3	2	3	W				
					2	2	2					
					4	2	3					
					1	3	2	A				
					2	4	3					
					2	3	2	A				
					0	2	2					
					2	2	2					
					1	4	3					
					4	2	3					
					2	4	4					
					2	4	4	W				
					0	3	3	A				
					0	2	4					
					3	4	3					
					1	3	4	WA				
					5	4	4					
					1	4	4	W				
					4	4	3					
					2	4	3					
					3	5	4	A				
					0	4	4					
					0	4	3					

.

	orc2-1				*orc2-2*				*orc2-3*			
	En	Ep	Su	*	En	Ep	Su	*	En	Ep	Su	*
Early Heart	5	4	3		2	4	4		20	2	2	
	3	4	3		0	5	3	AW	50	3	5	AW
	3	6	3	A	1	4	4		50	1	2	
	3	4	5		1	8	2	W	14	2	3	
	1	4	4		0	4	3		7	2	2	
	2	4	4	W	1	3	4	WA	50	3	3	A
	1	10	5	WA	1	4	2		33	1	2	
	11	4	5		1	3	3	A	18	2	2	
	5	7	5	WA	4	4	3	W	28	3	2	A
	7	4	2		3	4	3		12	1	2	
	3	6	4	WA	2	4	2					
	2	6	4		0	4	3	W				
	1	5	5	A	3	4	4					
					2	4	3	W				
					1	4	3					
					1	4	3					
					2	4	3					
Heart	1	12	5	A	1	8	6		45	2	2	
	8	12	5		1	8	5		25	1	1	
	3	8	6	W	4	2	3	W	7	4	8	
	9	4	5		3	7	4	WA	30	2	1	
	3	6	5	A	1	8	4	W	70	2	3	
	0	8	7	WA	3	2	2		30	2	6	
	7	5	5	A	0	4	3		30	1	2	
	2	6	5	AW	2	4	3	W	30	2	2	
	3	3	5	A	1	2	5		4	2	2	
	2	4	3		1	4	2		40	2	2	
	4	6	5	WA	0	5	3	WA	40	3	3	A
	2	3	6	A	2	4	3		45	2	5	
	4	2	7	W	1	4	4	W	35	2	3	
	8	8	4		3	4	3		20	1	3	
	0	3	3	A	1	4	4					
					0	4	3	W				
					0	4	3					
					0	2	3					
Late Heart	2	5	4	A	0	4	4		70	2	7	
	2	4	5	W	4	7	3	WA	40	4	4	
	0	4	4	W	2	8	5		40	3	3	A
	5	7	6	A	2	2	4		40	2	1	
	5	4	6	W	3	8	3	W	70	2	2	
	6	8	6		3	3	6	A	35	1	1	
	3	6	8	A	1	7	3	WA	5	2	6	
	5	4	5	W	1	3	5	A	50	2	4	
	2	8	5	W	2	4	2		5			
	2	7	5	AW	1	5	5	A	5			
	2	5	4	A	3	4	2		33	4		
	6	12	4	AW	0	6	4	A	15			
	0	6	3	A	0	5	3	A	10	1	3	
					0	4	3		40	2	4	
									45			
									70	3	6	A
									6			

6. REFERENCES

Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics International **11:** 36-42

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653-657

Andrade RP, Pascoal S, Palmeirim I (2005) Thinking clockwise. Brain Res Brain Res Rev **49:** 114-119

Araki M, Wharton RP, Tang Z, Yu H, Asano M (2003) Degradation of origin recognition complex large subunit by the anaphase-promoting complex in Drosophila. Embo J **22:** 6115-6126

Asano M, Wharton RP (1999) E2F mediates developmental and cell cycle regulation of ORC1 in Drosophila. Embo J **18:** 2435-2448

Auth T, Kunkel E, Grummt F (2006) Interaction between HP1alpha and replication proteins in mammalian cells. Exp Cell Res **312:** 3349-3359

Bajon C, Horlow C, Motamayor JC, Sauvanet A, Robert D (1999) Megasporogenesis in Arabidopsis thaliana L.: an ultrastructural study. Sex Plant Reprod **12:** 99-109

Barker RF, Harberd NP, Jarvis MG, Flavell RB (1988) Structure and evolution of the intergenic region in a ribosomal DNA repeat unit of wheat. J Mol Biol **201:** 1-17

Baroux C, Fransz P, Grossniklaus U (2004) Nuclear fusions contribute to polyploidization of the gigantic nuclei in the chalazal endosperm of Arabidopsis. Planta **220:** 38-46

Baroux C, Gagliardini V, Page DR, Grossniklaus U (2006) Dynamic regulatory interactions of Polycomb group genes: MEDEA autoregulation is required for imprinted gene expression in Arabidopsis. Genes Dev **20:** 1081-1086

Baud S, Bellec Y, Miquel M, Bellini C, Caboche M, Lepiniec L, Faure JD, Rochat C (2004) gurke and pasticcino3 mutants affected in embryo development are impaired in acetyl-CoA carboxylase. EMBO Rep **5:** 515-520

Baulcombe D (2005) RNA silencing. Trends Biochem Sci **30:** 290-293

Bauwens S, Van Oostveldt P, Engler G, Van Montagu M (1991) Distribution of the rDNA and three classes of highly repetitive DNA in the chromatin of interphase nuclei of Arabidopsis thaliana. Chromosoma **101:** 41-48

Bell SP (2002) The origin recognition complex: from simple origins to complex functions. Genes Dev **16:** 659-672

Bell SP, Kobayashi R, Stillman B (1993) Yeast origin recognition complex functions in transcription silencing and DNA replication. Science **262:** 1844-1849

Bell SP SB (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature **357(6374):** 128-134

Boavida LC, Becker JD, Feijo JA (2005) The making of gametes in higher plants. Int J Dev Biol **49:** 595-614

Boisnard-Lorig C, Colon-Carmona A, Bauch M, Hodge S, Doerner P, Bancharel E, Dumas C, Haseloff J, Berger F (2001) Dynamic analyses of the expression of the

HISTONE::YFP fusion protein in arabidopsis show that syncytial endosperm is divided in mitotic domains. Plant Cell **13:** 495-509

Bonner RF, Emmert-Buck M, Cole K, Pohida T, Chuaqui R, Goldstein S, Liotta LA (1997) Laser capture microdissection: molecular analysis of tissue. Science **278:** 1481,1483

Brand L, Horler M, Nuesch E, Vassalli S, Barrell P, Yang W, Jefferson RA, Grossniklaus U, Curtis MD (2006) A versatile and reliable two-component system for tissue-specific gene induction in Arabidopsis. Plant Physiol **141:** 1194-1204

Brandes A, Thompson H, Dean C, Heslop-Harrison JS (1997) Multiple repetitive DNA sequences in the paracentromeric regions of Arabidopsis thaliana L. Chromosome Res **5:** 238-246

Brukhin V, Gheyselinck J, Gagliardini V, Genschik P, Grossniklaus U (2005) The RPN1 subunit of the 26S proteasome in Arabidopsis is essential for embryogenesis. Plant Cell **17:** 2723-2737

Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O, Bouchez D (2002) The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell **14:** 833-845

Carmichael JS, Friedman WE (1995) Double Fertilization in Gnetum gnemon: The Relationship between the Cell Cycle and Sexual Reproduction. Plant Cell **7:** 1975-1988

Chanvivattana Y, Bishopp A, Schubert D, Stock C, Moon YH, Sung ZR, Goodrich J (2004) Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. Development **131:** 5263-5276

Chaudhury AM, Ming L, Miller C, Craig S, Dennis ES, Peacock WJ (1997) Fertilization-independent seed development in Arabidopsis thaliana. Proc Natl Acad Sci U S A **94:** 4223-4228

Chen S, Hofius D, Sonnewald U, Bornke F (2003) Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. Plant J **36:** 731-740

Chesnokov IN, Chesnokova ON, Botchan M (2003) A cytokinetic function of Drosophila ORC6 protein resides in a domain distinct from its replication activity. Proc Natl Acad Sci U S A **100:** 9150-9155

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16:** 735-743

Collinge MA, Spillane C, Kohler C, Gheyselinck J, Grossniklaus U (2004) Genetic interaction of an origin recognition complex subunit and the Polycomb group gene MEDEA during seed development. Plant Cell **16(4):** 1035-1046

Colon-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Technical advance: spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant J **20:** 503-508

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133:** 462-469

Cuvier O, Lutzmann M, Mechali M (2006) ORC is necessary at the interphase-to-mitosis transition to recruit cdc2 kinase and disassemble RPA foci. Curr Biol **16:** 516-523

Cvetic C, Walter JC (2005) Eukaryotic origins of DNA replication: could you please be more specific? Semin Cell Dev Biol **16:** 343-353

DePamphilis ML (2000) Review: nuclear structure and DNA replication. J Struct Biol **129:** 186-197

DePamphilis ML (2003) The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. Gene **310:** 1-15

DePamphilis ML (2005) Cell cycle dependent regulation of the origin recognition complex. Cell Cycle **4:** 70-79

DePamphilis ML, Blow JJ, Ghosh S, Saha T, Noguchi K, Vassilev A (2006) Regulating the licensing of DNA replication origins in metazoa. Curr Opin Cell Biol **18:** 231-239

Dhar SK, Dutta A (2000) Identification and characterization of the human ORC6 homolog. J Biol Chem **275:** 34983-34988

Dhonukshe P, Vischer N, Gadella TW, Jr. (2006) Contribution of microtubule growth polarity and flux to spindle assembly and functioning in plant cells. J Cell Sci **119:** 3193-3205

Diaz-Trivino S, del Mar Castellano M, de la Paz Sanchez M, Ramirez-Parra E, Desvoyes B, Gutierrez C (2005) The genes encoding Arabidopsis ORC subunits are E2F targets and the two ORC1 genes are differently expressed in proliferating and endoreplicating cells. Nucleic Acids Res **33:** 5404-5414

Dillin A, Rine J (1998) Roles for ORC in M phase and S phase. Science **279:** 1733-1737

Doelling JH, Yan N, Kurepa J, Walker J, Vierstra RD (2001) The ubiquitin-specific protease UBP14 is essential for early embryo development in Arabidopsis thaliana. Plant J **27:** 393-405

Dresselhaus T, Srilunchang KO, Leljak-Levanic D, Schreiber DN, Garg P (2006) The fertilization-induced DNA replication factor MCM6 of maize shuttles between cytoplasm and nucleus, and is essential for plant growth and development. Plant Physiol **140:** 512-527

Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA (1996) Laser capture microdissection. Science **274:** 998-1001

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391:** 806-811

Francis D (2007) The plant cell cycle - 15 years on. New Phytol **174:** 261-278

Fransz P, Armstrong S, Alonso-Blanco C, Fischer TC, Torres-Ruiz RA, Jones G (1998) Cytogenetics for the model system Arabidopsis thaliana. Plant J **13:** 867-876

Fransz P, Soppe W, Schubert I (2003) Heterochromatin in interphase nuclei of Arabidopsis thaliana. Chromosome Res **11:** 227-240

Franzmann L, Patton DA, Meinke DW (1989) In vitro morphogenesis of arrested embryos from lethal mutants of Arabidopsis thaliana. Theor Appl Genet **77**

Friedman WE (1991) Double fertilization in Ephedra trifurca, a non-flowering seed plant: The relationship between fertilization events and the cell cycle. Protoplasma **165:** 106-120

Friedman WE (1999) Expression of the cell cycle in sperm of Arabidopsis: implications for understanding patterns of gametogenesis and fertilization in plants and other eukaryotes. Development **126:** 1065-1075

Gaudin V, Libault M, Pouteau S, Juul T, Zhao G, Lefebvre D, Grandjean O (2001) Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis. Development **128:** 4847-4858

Ge SJ, Yao XL, Yang ZX, Zhu ZP (1998) An Arabidopsis embryonic lethal mutant with reduced expression of alanyl-tRNA synthetase gene. Cell Res **8:** 119-134

Glover DM (1989) Mitosis in Drosophila. J Cell Sci **92 (Pt 2):** 137-146

Gomez LD, Baud S, Gilday A, Li Y, Graham IA (2006) Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46:** 69-84

Goubet F, Misrahi A, Park SK, Zhang Z, Twell D, Dupree P (2003) AtCSLA7, a cellulose synthase-like putative glycosyltransferase, is important for pollen tube growth and embryogenesis in Arabidopsis. Plant Physiol **131:** 547-557

Grossniklaus U, Vielle-Calzada JP, Hoeppner MA, Gagliano WB (1998) Maternal control of embryogenesis by MEDEA, a polycomb group gene in Arabidopsis. Science **280:** 446-450

Guignard L (1899) Sur les antherozyides et la double copulation
sexuelle chez les vйgйtaux angiosperms. Rev Gen Bot **11:** 129-135

Guitton AE, Berger F (2005) Control of reproduction by Polycomb Group complexes in animals and plants. Int J Dev Biol **49:** 707-716

Guitton AE, Page DR, Chambrier P, Lionnet C, Faure JE, Grossniklaus U, Berger F (2004) Identification of new members of Fertilisation Independent Seed Polycomb Group pathway involved in the control of seed development in Arabidopsis thaliana. Development **131:** 2971-2981

Guo HS, Fei JF, Xie Q, Chua NH (2003) A chemical-regulated inducible RNAi system in plants. Plant J **34:** 383-392

Haberer G, Fischer TC, Torres-Ruiz RA (1996) Mapping of the nucleolus organizer region on chromosome 4 in Arabidopsis thaliana. Mol Gen Genet **250:** 123-128

Haecker A, Gross-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T (2004) Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development **131:** 657-668

Haseloff J (1999) GFP variants for multispectral imaging of living cells. Methods Cell Biol **58:** 139-151

Helliwell C, Waterhouse P (2003) Constructs and methods for high-throughput gene silencing in plants. Methods **30:** 289-295

Helliwell CA, Waterhouse PM (2005) Constructs and methods for hairpin RNA-mediated gene silencing in plants. Methods Enzymol **392:** 24-35

Higashiyama T, Yabe S, Sasaki N, Nishimura Y, Miyagishima S, Kuroiwa H, Kuroiwa T (2001) Pollen tube attraction by the synergid cell. Science **293:** 1480-1483

Holding DR, Springer PS (2002) The Arabidopsis gene PROLIFERA is required for proper cytokinesis during seed development. Planta **214:** 373-382

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol **5:** R85

Huang Z, Zang K, Reichardt LF (2005) The origin recognition core complex regulates dendrite and spine development in postmitotic neurons. J Cell Biol **170:** 527-535

Inagaki S, Suzuki T, Ohto MA, Urawa H, Horiuchi T, Nakamura K, Morikami A (2006) Arabidopsis TEBICHI, with helicase and DNA polymerase domains, is required for regulated cell division and differentiation in meristems. Plant Cell **18:** 879-892

Ingouff M, Jullien PE, Berger F (2006) The female gametophyte and the endosperm control cell proliferation and differentiation of the seed coat in Arabidopsis. Plant Cell **18:** 3491-3501

Ishige F, Takaichi M, Foster R, Chua NH, Oeda K (1999) A G-box motif (GCCACGTGCC) tetramer confers high-level constitutive expression in dicot and monocot plants. Plant J **18:** 443-448

Jenik PD, Jurkuta RE, Barton MK (2005) Interactions between the cell cycle and embryonic patterning in Arabidopsis uncovered by a mutation in DNA polymerase epsilon. Plant Cell **17:** 3362-3377

Jurgens G (1992) Pattern formation in the flowering plant embryo. Curr Opin Genet Dev **2:** 567-570

Jurgens G, Mayer U, Busch M, Lukowitz W, Laux T (1995) Pattern formation in the Arabidopsis embryo: a genetic perspective. Philos Trans R Soc Lond B Biol Sci **350:** 19-25

Kearsey SE, Cotterill S (2003) Enigmatic variations: divergent modes of regulating eukaryotic DNA replication. Mol Cell **12:** 1067-1075

Kohler C, Hennig L, Bouveret R, Gheyselinck J, Grossniklaus U, Gruissem W (2003) Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development. Embo J **22:** 4804-4814

Koltunow AM, Grossniklaus U (2003) Apomixis: a developmental perspective. Annu Rev Plant Biol **54:** 547-574

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls tissue-specific expression of chimeric genes carried by a novel type of Agrobacterium binary vector. Molecular and General Genetics **204:** 383-396

Kowalski SP, Lan TH, Feldmann KA, Paterson AH (1994) Comparative mapping of Arabidopsis thaliana and Brassica oleracea chromosomes reveals islands of conserved organization. Genetics **138:** 499-510

Lal SK, Hannah LC (1999) Maize transposable element Ds is differentially spliced from primary transcripts in endosperm and suspension cells. Biochem Biophys Res Commun **261:** 798-801

Larsson AS, Landberg K, Meeks-Wagner DR (1998) The TERMINAL FLOWER2 (TFL2) gene controls the reproductive transition and meristem identity in Arabidopsis thaliana. Genetics **149:** 597-605

Liu Cm CM, McElver J, Tzafrir I, Joosen R, Wittich P, Patton D, Van Lammeren AA, Meinke D (2002) Condensin and cohesin knockouts in Arabidopsis exhibit a titan seed phenotype. Plant J **29:** 405-415

Liu CM, Meinke DW (1998) The titan mutants of Arabidopsis are disrupted in mitosis and cell cycle control during seed development. Plant J **16:** 21-31

Liu Z, Makaroff CA (2006) Arabidopsis separase AESP is essential for embryo development and the release of cohesin during meiosis. Plant Cell **18:** 1213-1225

Lopes MA, Larkins BA (1993) Endosperm origin, development, and function. Plant Cell **5:** 1383-1399

Loupart ML KS, Heck MS. (2000) Aberrant replication timing induces defective chromosome condensation in Drosophila ORC2 mutants. Current Biology **10(24):** 1547-1556

Mac Auley A, Werb Z, Mirkes PE (1993) Characterization of the unusually rapid cell cycles during rat gastrulation. Development **117:** 873-883

MacAuley A, Cross JC, Werb Z (1998) Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. Mol Biol Cell **9:** 795-807

Martienssen RA (1998) Functional genomics: probing plant gene function and expression with transposons. Proc Natl Acad Sci U S A **95:** 2021-2026

Martinez-Zapater J, Estelle MA, Somerville CR (1986) A highly repeated DNA sequence in Arabidopsis thaliana. Mol. Gen. Genet. **204:** 417-423

Masuda HP, Ramos GB, de Almeida-Engler J, Cabral LM, Coqueiro VM, Macrini CM, Ferreira PC, Hemerly AS (2004) Genome based identification and analysis of the pre-replicative complex of Arabidopsis thaliana. FEBS Lett **574:** 192-202

Mayama T, Ohtsubo E, Tsuchimoto S (2003) Isolation and expression analysis of petunia CURLY LEAF-like genes. Plant Cell Physiol **44:** 811-819

Mayer U, Herzog U, Berger F, Inze D, Jurgens G (1999) Mutations in the pilz group genes disrupt the microtubule cytoskeleton and uncouple cell cycle progression from cell division in Arabidopsis embryo and endosperm. Eur J Cell Biol **78:** 100-108

McClinton RS, Sung ZR (1997) Organization of cortical microtubules at the plasma membrane in Arabidopsis. Planta **201:** 252-260

McElroy D, Chamberlain DA, Moon E, Wilson KJ (1995) Development ofgusA reporter gene constructs for cereal transformation: Availability of plant transformation vectors from the CAMBIA Molecular Genetic Resource Service. Molecular Breeding **1:** 27-37

McElver J, Patton D, Rumbaugh M, Liu C, Yang LJ, Meinke D (2000) The TITAN5 gene of Arabidopsis encodes a protein related to the ADP ribosylation factor family of GTP binding proteins. Plant Cell **12:** 1379-1392

McGrath JM, Jangso MM, Pichersky E (1993) Duplicate sequences with a similarity to expressed genes in the genome of Arabidopsis thaliana. Theor. Appl. Genet. **86:** 880-888

Meinke DW (1985) Embryo-lethal mutants of Arabidopsis thaliana: Analysis of mutants with a wide range of lethal phases. Theor Appl Genet **69:** 543-552

Mendez J, Zou-Yang XH, Kim SY, Hidaka M, Tansey WP, Stillman B (2002) Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell **9:** 481-491

Meyerowitz E (1994) Structure and organization of the Arabidopsis thaliana genome. In: Meyerowitz EM, Somerville CR, eds. Arabidopsis. New York: Cold Spring Harbor Laboratory Press.**:** 21-36

Micklem G RA, Harwood J, Nasmyth K, Diffley JF. (1993) Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. Nature **366(6450):** 87-89

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family of rice. Plant Physiol **138:** 1903-1913

Mogensen HL, Holm PB (1995) Dynamics of Nuclear DNA Quantities during Zygote Development in Barley. Plant Cell **7:** 487-494

Mogensen HL, Leduc N, Matthys-Rochon E, Dumas C (1995) Nuclear DNA amounts in the egg and zygote of maize (Zea Mays L.). Planta **197:** 641-645

Moore I, Samalova M, Kurup S (2006) Transactivated and chemically inducible gene expression in plants. Plant J **45:** 651-683

Moore JM, Calzada JP, Gagliano W, Grossniklaus U (1997) Genetic characterization of hadad, a mutant disrupting female gametogenesis in Arabidopsis thaliana. Cold Spring Harb Symp Quant Biol **62:** 35-47

Mori Y, Yamamoto T, Sakaguchi N, Ishibashi T, Furukawa T, Kadota Y, Kuchitsu K, Hashimoto J, Kimura S, Sakaguchi K (2005) Characterization of the origin recognition complex (ORC) from a higher plant, rice (Oryza sativa L.). Gene **353:** 23-30

Muehlbauer GJ, Fowler JE, Freeling M (1997) Sectors expressing the homeobox gene liguleless3 implicate a time-dependent mechanism for cell fate acquisition along the proximal-distal axis of the maize leaf. Development **124:** 5097-5106

Nawaschin S (1898) Resulte eine revision der Befruchtungsvorgange bei Lilium martagon und Ritillaria tenella. Bulletin of the Academy of Imperial Sciences St. Petersburg **9:** 377-382

Nguyen VQ, Co C, Li JJ (2001) Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature **411:** 1068-1073

Ohad N, Margossian L, Hsu YC, Williams C, Repetti P, Fischer RL (1996) A mutation that allows endosperm development without fertilization. Proc Natl Acad Sci U S A **93:** 5319-5324

Ohta S, Tatsumi Y, Fujita M, Tsurimoto T, Obuse C (2003) The ORC1 cycle in human cells: II. Dynamic changes in the human ORC complex during the cell cycle. J Biol Chem **278:** 41535-41540

Ohtani K, DeGregori J, Leone G, Herendeen DR, Kelly TJ, Nevins JR (1996) Expression of the HsOrc1 gene, a human ORC1 homolog, is regulated by cell proliferation via the E2F transcription factor. Mol Cell Biol **16:** 6977-6984

Osmont KS, Sadeghian N, Freeling M (2006) Mosaic analysis of extended auricle1 (eta1) suggests that a two-way signaling pathway is involved in positioning the blade/sheath boundary in Zea mays. Dev Biol **295:** 1-12

Page DR, Grossniklaus U (2002) The art and design of genetic screens: Arabidopsis thaliana. Nat Rev Genet **3:** 124-136

Pak DT, Pflumm M, Chesnokov I, Huang DW, Kellum R, Marr J, Romanowski P, Botchan MR (1997) Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. Cell **91:** 311-323

Perera RJ, Linard CG, Signer ER (1993) Cytosine deaminase as a negative selective marker for Arabidopsis. Plant Mol Biol **23:** 793-799

Pflumm MF (2002) The role of DNA replication in chromosome condensation. Bioessays **24:** 411-418

Pflumm MF BM (2001) Orc mutants arrest in metaphase with abnormally condensed chromosomes. Development **128(9):** 1697-1707

Pina C, Pinto F, Feijo JA, Becker JD (2005) Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant Physiol **138:** 744-756

Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B (2004) Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. Embo J **23:** 2651-2663

Prasanth SG, Prasanth KV, Stillman B (2002) Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. Science **297:** 1026-1031

Raynaud C, Perennes C, Reuzeau C, Catrice O, Brown S, Bergounioux C (2005) Cell and plastid division are coordinated through the prereplication factor AtCDT1. Proc Natl Acad Sci U S A **102:** 8216-8221

Ronceret A, Guilleminot J, Lincker F, Gadea-Vacas J, Delorme V, Bechtold N, Pelletier G, Delseny M, Chaboute ME, Devic M (2005) Genetic analysis of two Arabidopsis DNA polymerase epsilon subunits during early embryogenesis. Plant J **44:** 223-236

Roudier F, Fedorova E, Gyorgyey J, Feher A, Brown S, Kondorosi A, Kondorosi E (2000) Cell cycle function of a Medicago sativa A2-type cyclin interacting with a PSTAIRE-type cyclin-dependent kinase and a retinoblastoma protein. Plant J **23:** 73-83

Sambrook J, Russel D (2001) Molecular Cloning: A Laboratory Manual. 3rd Ed. Cold Spring Harbor Laboratory Press, New York.

Sauer M, Balla J, Luschnig C, Wisniewska J, Reinohl V, Friml J, Benkova E (2006) Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. Genes Dev **20:** 2902-2911

Shareef MM, King C, Damaj M, Badagu R, Huang DW, Kellum R (2001) Drosophila heterochromatin protein 1 (HP1)/origin recognition complex (ORC) protein is associated with HP1 and ORC and functions in heterochromatin-induced silencing. Mol Biol Cell **12:** 1671-1685

Shevell DE, Leu WM, Gillmor CS, Xia G, Feldmann KA, Chua NH (1994) EMB30 is essential for normal cell division, cell expansion, and cell adhesion in Arabidopsis and encodes a protein that has similarity to Sec7. Cell **77:** 1051-1062

Shimada K, Gasser SM (2007) The origin recognition complex functions in sister-chromatid cohesion in Saccharomyces cerevisiae. Cell **128:** 85-99

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature **407:** 319-320

Sorensen AM, Kroeber S, Saedler H (2004) The ABNORMAL GAMETOPHYTES (AGM) gene product of Arabidopsis demonstrates a role in mitosis during gamete development. Plant Cell Physiol **45:** 905-913

Springer PS, Holding DR, Groover A, Yordan C, Martienssen RA (2000) The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G(1) phase and is required maternally for early Arabidopsis development. Development **127:** 1815-1822

Springer PS, McCombie WR, Sundaresan V, Martienssen RA (1995) Gene trap tagging of PROLIFERA, an essential MCM2-3-5-like gene in Arabidopsis. Science **268:** 877-880

Steinborn K, Maulbetsch C, Priester B, Trautmann S, Pacher T, Geiges B, Kuttner F, Lepiniec L, Stierhof YD, Schwarz H, Jurgens G, Mayer U (2002) The Arabidopsis PILZ group genes encode tubulin-folding cofactor orthologs required for cell division but not cell growth. Genes Dev **16:** 959-971

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, Galweiler L, Palme K, Jurgens G (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science **286:** 316-318

Stougaard J (1993) Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. The Plant J. **3:** 755-761

Stuermer A, Hoehn K, Faul T, Auth T, Brand N, Kneissl M, Putter V, Grummt F (2007) Mouse pre-replicative complex proteins colocalise and interact with the centrosome. Eur J Cell Biol **86:** 37-50

Sun WH, Coleman TR, DePamphilis ML (2002) Cell cycle-dependent regulation of the association between origin recognition proteins and somatic cell chromatin. Embo J **21:** 1437-1446

Suter B, Tong A, Chang M, Yu L, Brown GW, Boone C, Rine J (2004) The origin recognition complex links replication, sister chromatid cohesion and transcriptional silencing in Saccharomyces cerevisiae. Genetics **167:** 579-591

Tanaka H, Dhonukshe P, Brewer PB, Friml J (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell Mol Life Sci **63:** 2738-2754

Tatsumi Y, Ohta S, Kimura H, Tsurimoto T, Obuse C (2003) The ORC1 cycle in human cells: I. cell cycle-regulated oscillation of human ORC1. J Biol Chem **278:** 41528-41534

Tian HQ, Yuan T, Russell SD (2005) Relationship between double fertilization and the cell cycle in male and female gametes of tobacco. Sexual Plant Reproduction **17:** 243-252

Torres-Ruiz RA, Jurgens G (1994) Mutations in the FASS gene uncouple pattern formation and morphogenesis in Arabidopsis development. Development **120:** 2967-2978

Torres-Ruiz RA, Lohner A, Jurgens G (1996) The GURKE gene is required for normal organization of the apical region in the Arabidopsis embryo. Plant J **10:** 1005-1016

Triolo T, Sternglanz R (1996) Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature **381:** 251-253

Tzafrir I, McElver JA, Liu Cm CM, Yang LJ, Wu JQ, Martinez A, Patton DA, Meinke DW (2002) Diversity of TITAN functions in Arabidopsis seed development. Plant Physiol **128:** 38-51

Vanstraelen M, Van Damme D, De Rycke R, Mylle E, Inze D, Geelen D (2006) Cell cycle-dependent targeting of a kinesin at the plasma membrane demarcates the division site in plant cells. Curr Biol **16:** 308-314

Vidwans SJ, DiGregorio PJ, Shermoen AW, Foat B, Iwasa J, Yakubovich N, O'Farrell PH (2002) Sister chromatids fail to separate during an induced endoreplication cycle in Drosophila embryos. Curr Biol **12:** 829-833

Vieten A, Vanneste S, Wisniewska J, Benkova E, Benjamins R, Beeckman T, Luschnig C, Friml J (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development **132:** 4521-4531

Walbot V, Evans MM (2003) Unique features of the plant life cycle and their consequences. Nat Rev Genet **4:** 369-379

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J **27:** 581-590

Wessler SR (1991) The maize transposable Ds1 element is alternatively spliced from exon sequences. Mol Cell Biol **11:** 6192-6196

Wessler SR, Baran G, Varagona M (1987) The maize transposable element Ds is spliced from RNA. Science **237:** 916-918

Willemse MTM, van Went JL (1984) The female gametophyte. In: Johri BM, ed. Embryology of angiosperms, Berlin, Germany: Springer Verlag

Witmer X, Alvarez-Venegas R, San-Miguel P, Danilevskaya O, Avramova Z (2003) Putative subunits of the maize origin of replication recognition complex ZmORC1-ZmORC5. Nucleic Acids Res **31:** 619-628

Xu C, Fan J, Froehlich JE, Awai K, Benning C (2005) Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in Arabidopsis. Plant Cell **17:** 3094-3110

Yadegari R, Paiva G, Laux T, Koltunow AM, Apuya N, Zimmerman JL, Fischer RL, Harada JJ, Goldberg RB (1994) Cell Differentiation and Morphogenesis Are Uncoupled in Arabidopsis raspberry Embryos. Plant Cell **6:** 1713-1729

Yakovlev MS, Alimova G.K. (1976) Embryogenesis in Arabidopsis thaliana (L.) Heynh. (Cruciferae). Bot. Zhurnal (Russ) **61:** 12-24

Zuo J, Niu QW, Chua NH (2000) Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J **24:** 265-273